REMARKS

I. Status of the Claims

With entry of this Amendment, claims 47, 61, 63-68, 78, 79, and 83 are pending in the application. All of the claims stand rejected.

Solely in an effort to expedite prosecution, Applicants have amended claim 47 to recite that the method comprises stereotaxically administering into the central nervous system an implant comprising an extracellular matrix and cells **human** infected by a replication defective recombinant adenovirus that encodes human superoxide dismutase. Support for the proposed amendment is found in the specification, including, for example, at page 16, lines 9-13; page 17, lines 2-9; page 18, line 14 through page 20, line 6; and page 6, line 24 through page 7, line 6. Applicants have canceled claim 62. This amendment does not add any new matter.

II. The Specification Enables the Full Scope of the Claims

For the reasons of record, the Office maintains the rejection of claims 47, 61-68, 78, 79, and 83 under 35 U.S.C. § 112, first paragraph, as allegedly containing subject matter that was not described in the specification in such a way as to enable one skilled in the art to make or use the invention without undue experimentation. Office Action, mailed September 30, 2004, page 2. Applicants respectfully traverse the rejection.

The relevant inquiry for enablement is whether one reasonably skilled in the art could make and/or use the invention from the disclosures in the specification, coupled with information known in the art, without undue experimentation. M.P.E.P. § 2164.01, 2100-185 (Rev. May 2004). Factors to be considered when determining whether experimentation is undue include but are not limited to:

(a) The breadth of the claims;

(b) The nature of the invention;

(c) The state of the prior art;

(d) The level of one of ordinary skill;

(e) The level of predictability in the art;

(f) The amount of direction provided by the inventor;

(g) The existence of working examples; and

(h) The quantity of experimentation needed to make or use the invention based on the content of the disclosure. *Id.* at § 2164.01(a), 2100-185.

The Office addressed several of these factors in the Office Action mailed February 25, 2004. The Office's central argument appearing in its discussion of each *Wands* factor is the issue of sustaining therapeutic levels of expression of the superoxide dismutase gene. The Office argues that sustained expression of the superoxide dismutase gene could not be achieved due to an immunological response to the adenovirus. Taking into account each of the factors considered by the Office, Applicants submit that the specification enables the claims as amended.

A. Nature of the Invention

In the Action mailed February 25, 2004, the Office begins its analysis by examining the nature of the invention, noting that "the invention claims a method whereby a gene encoding a superoxide dismutase (SOD-1) is used to treat a variety of chronic diseases." Office Action mailed February 25, 2004, page 3. This is not an accurate characterization of the invention. Applicants note that the claims were

amended in the previous response and are now directed to a method of treating a single disease: Parkinson's disease.

The Office then concludes that:

> Because the invention claims a method of treatment of these chronic diseases, the method necessarily requires the ability to sustain therapeutic levels of expression of the superoxide dismutase gene. This is because the chronic nature of the diseases requires a sustained expression in order to treat the disease for the length of the patient's affliction therewith.

Id.

Again the Office mischaracterizes the claimed invention. Without support, the Office summarily concludes that the term treatment requires "sustained expression" of therapeutic levels of the superoxide dismutase gene that endures for the length of the patient's affliction with a particular disease. In essence, the Office requires Applicants to enable a method of curing Parkinson's disease. But that is not what Applicants have claimed. Applicants' claims are directed to methods of treating Parkinson's disease and cover any therapeutic effect resulting from the administration of the cells infected with the claimed adenovirus. This is consistent with the ordinary meaning of the term treatment. For example, the American Heritage dictionary (4th Edition) defines treatment as: "to give medical aid to someone; to give medical aid to counteract (a disease or condition)." In other words, Applicants' claims cover methods of mitigating the effects of Parkinson's disease and may be used alone or in conjunction with other therapies. The Office provides no evidence to support its construction that a method of treatment requires sustained expression of therapeutic levels of superoxide dismutase to treat the disease for the length of the patient's affliction. Nor is there any such support.

Thus, placed in proper perspective, the invention is directed to a method of treating Parkinson's disease and is not broadly drawn to treating any disease. In addition, the claims cover methods of mitigating the effects of Parkinson's disease—not methods of curing Parkinson's disease through sustained expression of the superoxide dismutase gene.

B. Scope of the Invention

Next, the Office addresses the scope of the invention, stating only that "[t]he scope of the invention is very broad, encompassing the ability to treat a large number of chronic diseases." Office Action mailed February 25, 2004, page 3. Again, as noted above, the scope of the invention is not nearly so broad and has been amended to cover methods of treating one disease: Parkinson's disease.

C. State of the Art

Regarding the state of the art, the Office states that "gene therapy, as a whole is an unpredictable area." *Id.* at 4. According to the Office, "[t]his is especially true with regard to the use of adenovirus to sustain the expression of a gene at therapeutic levels." *Id.* As before, the Office bases its non-enablement argument on the issue of whether Applicants have enabled a method for sustaining gene expression at therapeutic levels, a recitation not found in the pending claims.

The specification enables one of skill in the art to make and use the claimed invention without undue experimentation. In particular, the specification enables one of skill in the art to administer a superoxide dismutase gene to a patient with Parkinson's disease and to express the gene in the patient at levels sufficient to mitigate the effects of the disease. For example, the specification teaches how to make adenoviral vectors

that can be used in the claimed method. Specification, pages 10-14 and 23-25. In addition, the specification teaches how to administer the adenoviral vector to a patient. Specification, pages 14-20. More specifically, the specification teaches stereotaxically administering the adenoviral vector. Specification, page 16, lines 9-13. The specification also teaches that the vectors can be administered to a patient *ex vivo* using an implant comprising cells infected with the adenoviral vector and an extracellular matrix. Specification, pages 18-20.

As further evidence that the specification enables the full scope of the claimed method, Applicants submit an article by Barkats *et al.* entitled "Intrastriatal Grafts of Embryonic Mesencephalic Rat Neurons Genetically Modified Using an Adenovirus Encoding Human Cu/Zn Superoxide Dismutase."[1] ("Barkats-1997.") This article was accepted for publication in September 1996, and published in May 1997.

In the response dated July 26, 2004, Applicants submitted a later reference, Barkats-1998, as evidence that the specification enabled the claimed method. Barkats-1998 is a review article that refers to the underlying experimental work described in Barkats-1997. The Office objected to Barkats-1998 and stated that "applicants cannot rely on post-filing art to supplement the teachings of the instant specification." Office Action mailed September 30, 2004, page 4. But Applicants do not rely on Barkats-1998 or Barkats-1997 to supplement the teachings of the specification. Rather, they offer those references as additional evidence that the specification, as filed, enables the claimed invention. It is well settled that a publication dated after the filing date of an application may be offered as evidence that the disclosed device would have been

[1] Barkats *et al.*, Neuroscience, 78(3):703-13 (1997) (Exhibit 1).

operative. *Gould v. Quigg*, 822 F.2d 1074, 1078, 3 U.S.P.Q.2d 1302, 1305 (Fed. Cir. 1987).

Using the teachings of Applicants' specification, Barkats-1997 demonstrates that administering cells infected with an adenovirus encoding human superoxide dismutase improved the survival and functional efficacy of dopaminergic cells in a rat model for Parkinson's disease. For example, the vectors used in Barkats-1997 are disclosed in the specification. As discussed at page 704 under the Experimental Procedures section, Barkats-1997 used a recombinant, replication defective adenovirus containing the human copper-zinc superoxide dismutase gene, as disclosed in the specification. Specification, pages 11 and 23. The superoxide dismutase was inserted into a shuttle vector downstream from the long terminal repeat (LTR) of the Rous Sarcoma Virus (RSV), as disclosed in the specification. *Id.*

Next, the shuttle vector was co-transfected into the 293 cell line with a deficient adenoviral vector that had been linearized with the restriction enzyme, *ClaI*. Barkats-1997, page 704, col. 1, last paragraph. This procedure is disclosed in the specification, including, for example, at pages 13-14 and in Example 2. Defective, recombinant adenovirus was purified from the 293 cells and used to infect cells prior to stereotaxically implanting the infected cells into the central nervous systems of rats, according to a technique known in the art and previously described by Frodl *et al.*, Brain Research, 647:286-98 (1994) (Exhibit 2). Barkats-1997, page 704, col. 2, last full paragraph. The specification similarly teaches the infection of mammalian cells with defective, recombinant adenovirus and the subsequent stereotaxic administration of the infected cells into the central nervous system. Specification, pages 16-20. The

expression of superoxide dismutase in the brain was observed four days after cells were transplanted and persisted for at least 5 weeks. Barkats-1997, page 709, col. 1. In addition, five weeks after transplanting the cells into the central nervous system, significantly faster and more extensive functional recovery was observed in the rats expressing superoxide dismutase. *Id.* at 710, col. 2. Thus, Barkats-1997 and Barkats-1998 provide further evidence that Applicants' specification enables one of skill in the art to treat Parkinson's disease through *ex vivo* administration of cells infected with an adenovirus containing a DNA sequence encoding human superoxide dismutase.

The Office acknowledges that cells expressing superoxide dismutase in Barkats-1998 showed a greater survival rate following transplantation than control cells. Office Action mailed September 30, 2004, pages 4-5. But the Office dismissed those results because the differences in graft survival were not statistically significant. *Id.* at 5. Even if the graft survival was not statistically significant, Barkats-1997 notes that "there was a trend for a better survival of TH-immunoreactive grafted cells derived from hCuZnSOD-infected cell suspensions." Barkats-1997, page 709, col. 2. That finding is particularly significant because it shows that infection of cells *in vitro* with adenovirus prior to transplantation into the central nervous system does not reduce transplant survival or function. *Id.* at 709, cols. 1 and 2.

Moreover, graft survival is not the only measure of the efficacy of *ex vivo* gene delivery in the Parkinson's disease rat model used in Barkats-1997. For example, efficacy was also measured using a functional, rotational behavior assay. Barkats-1997, page 705, col. 2. In this functional assay, at five weeks after surgery, "the rotation score was significantly reduced in the SOD group compared to the control and βgal

groups." *Id.* And "[o]nly the SOD group exhibited complete reversal of rotation asymmetry and displayed a mean score that was significantly reduced compared to pre-transplantation and two weeks post-transplantation values." *Id.* Thus, administration of the superoxide dismutase gene demonstrated a statistically significant functional effect in this rat model.

Finally, the Office criticizes Applicants for relying on Barkats-1998, alleging that the claims were not commensurate in scope with the teachings of the reference. The Office noted that Barkats-1998 discloses an *ex vivo* method where cells are transfected with the adenovirus *in vitro* and then the infected cells are transplanted into a rat. Office Action mailed September 30, 2004, page 5. Addressing an alleged area of unpredictability related to gene therapy (i.e., immunogenic response to the adenovirus), the Office acknowledged that "[u]nder such circumstances, one would expect to see only a minimal immunogenic response . . . ." *Id.* The Office compared this to direct administration of the adenovirus, which allegedly "results in an immunogenic response, as evidenced by the teachings of Anderson, Verma, and Mountain." *Id.*

In an effort to expedite prosecution, Applicants have amended the claims to recite that the method comprises administering "an implant which comprises an extracellular matrix and human cells infected by a replication defective, recombinant adenovirus . . . ." Thus, the Office's assertions about low expression and immunogenicity based on the teachings of *Anderson*, *Verma*, and *Mountain* are not germane to the claimed methods.

D. Amount of Direction Provided by Applicants and Working Examples

The Office asserts that the specification does not provide any guidance or working examples. Office Action mailed February 25, 2004, page 5. This is not true. As discussed above, the specification provides considerable guidance as well as working examples regarding how to make adenoviruses containing the superoxide dismutase gene. In addition, the specification teaches one of skill in the art how to administer such an adenovirus to a patient either directly or as part of an implant comprising cells infected with the adenovirus. Thus, the specification fully enables one of skill in the art to make and use the claimed invention without undue experimentation.

The Office argues that "[t]here is no indication that the vector described in the instant specification has any capacity to overcome the deficiencies of using Ad vectors for gene therapy, as set forth in [*Verma*, *Anderson*, and *Mountain*]." Office Action mailed February 25, 2004, page 6. The Office cites *Verma* and *Anderson* as evidence that adenoviral vectors have difficulty in producing sustained expression for more than 5-10 days due to an immunological response to the adenovirus vector. Office Action mailed February 25, 2004, page 4.

As discussed above, however, Applicants' claims are directed to methods of treating Parkinson's disease and cover therapeutic effects resulting from the administration of cells infected with the adenovirus. The claims do not require sustained expression of superoxide dismutase at therapeutic levels for a certain duration of time. Furthermore, Barkats-1997 provides specific evidence that following the teachings of the instant application, sustained expression of SOD was achieved for at least 5 weeks. And this expression was achieved with a minimal immunological response, which, as

acknowledged by the Office, would be expected when administering cells infected with an adenovirus vector. Accordingly, the Office's concerns regarding low expression levels caused by immunogenic responses to the adenovirus are not relevant to the enablement of the claimed invention.

E. Level of Predictability and Quantity of Experimentation Required

Once again, based on *Verma*, *Anderson*, and *Mountain*, the Office asserts that the art is unpredictable because the immunological response to the adenovirus vector would prevent sustained expression of the therapeutic gene. More specifically, the Office states:

> [T]here are no Ad vectors that can adequately sustain the expression of a therapeutic gene in a human. This is because of the elicitation of a strong immune response in the cells upon delivery of the vector, which subsequently results in decreased expression of the therapeutic gene. This is particularly important as it regards the diseases indicated as being treated in the instant claims because these diseases, being chronic in nature, require a long-term method of expressing the therapeutic gene. Thus, in order to practice the claimed invention, the skilled artisan would need to first develop an Ad viral vector that was capable of expressing a therapeutic gene at sustained levels sufficient to treat a chronic disease.

Office Action mailed February 25, 2004, page 6.

As discussed above, however, the claims are directed to methods of administering cells infected with an adenovirus. Even the Office admits that one of skill in the art would expect only a minimal immune response to the adenovirus in such an *ex vivo* method. In addition, Barkats-1997 provides specific evidence that following the teachings of the instant application, sustained expression of SOD was achieved for at least 5 weeks and was not adversely affected by an immunological response to the adenovirus. The Office provides no other basis for arguing that the art was unpredictable or that the claimed invention would require undue experimentation.

Accordingly, for the reasons discussed above, Applicants respectfully submit that the specification provides an enabling disclosure that is commensurate in scope with the claimed subject matter.

III. Conclusion

In view of the foregoing, Applicants respectfully request that the Office reconsider and withdraw the enablement rejections of the pending claims and allow all pending claims.

Please grant any extensions of time required to enter this amendment and response and charge any additional required fees to our Deposit Account No. 06-0916.

Respectfully submitted,

FINNEGAN, HENDERSON, FARABOW,
GARRETT & DUNNER, L.L.P.

Dated: December 29, 2004

By: _____

Timothy B. Donaldson
Reg. No. 43,592
Tel: 571-203-2712
Fax: 202-408-4400

Pergamon

PII: S0306-4522(96)00526-X

Neuroscience Vol. 78, No. 3, pp. 703–713, 1997

0306–4522/97 $17.00+0.00

INTRASTRIATAL GRAFTS OF EMBRYONIC MESENCEPHALIC RAT NEURONS GENETICALLY MODIFIED USING AN ADENOVIRUS ENCODING HUMAN Cu/Zn SUPEROXIDE DISMUTASE

M. BARKATS,* N. NAKAO,† E. M. GRASBON-FRODL,† A. BILANG-BLEUEL,* F. REVAH,‡ J. MALLET*§ and P. BRUNDIN†

*Laboratoire de Génétique Moléculaire de la Neurotransmission et des Processus Neurodégénératifs, UMR CNRS C9923, Hôpital de la Pitié Salpêtrière, Paris, France

†Section for Neuronal Survival, Department of Physiology and Neuroscience, University of Lund, Lund, Sweden

‡Gencell Rhône-Poulenc-Rorer, Vitry sur Seine, France

Abstract—Intrastriatal grafting of embryonic dopamine-containing neurons is a promising approach for treating clinical and experimental Parkinson's disease. However, neuropathological analyses of grafted patients and transplanted rats have demonstrated that the survival of grafted dopamine neurons is relatively poor. In the present study, we pursued a strategy of transferring a potentially neuroprotective gene into rat embryonic mesencephalic rat cells *in vitro*, before grafting them into the denervated striatum of 6-hydroxydopamine-lesioned rats. We performed intrastriatal grafts of embryonic day 14 mesencephalic cells infected with replication-defective adenoviruses bearing either the human copper-zinc superoxide dismutase gene or, as a control, the *E. coli* lac Z marker gene. The transgenes were expressed in the grafts four days after transplantation and the expression persisted for at least five weeks thereafter. After five weeks postgrafting, there was more extensive functional recovery in the superoxide dismutase group as compared to the control (uninfected cells) and β-galactosidase groups. The functional recovery was significantly correlated with the number of tyrosine hydroxylase-positive cells in the grafts, although the clear trend to increased survival of the dopamine neurons in the superoxide dismutase grafts did not reach statistical significance.

Only a moderate inflammatory reaction was revealed by OX-42 immunostaining in all groups, suggesting that *ex vivo* gene transfer using adenoviral vectors is a promising method for delivering functional proteins into brain grafts. © 1997 IBRO. Published by Elsevier Science Ltd.

Key words: neural graft, mesencephalic cells, superoxide dismutase, adenovirus, *ex vivo* gene transfer, Parkinson's disease.

Transplants of dopamine-containing neurons into the brain of patients with Parkinson's disease (PD) have been found to survive and provide marked amelioration of motor symptoms. However, the symptomatic relief is far from complete and one possible contributing factor to the limited effect is poor survival of the implanted neurons.[21,29] It is estimated that only 5–20% of grafted dopamine neurons survive the transplantation procedure.[8,15,24,25,41] Therefore, several studies have focused on developing new strategies for enhancing the viability of embryonic dopamine neurons after transplantation into animals with experimental brain lesions.[22,24,36,42,46,50]

Because dopamine neurons can generate free radicals by autoxidation or monoamine oxidase-mediated metabolism of dopamine,[28] they may be particularly susceptible to damage by oxidative stress. Oxygen free radical production may increase during preparation and implantation of the graft tissue due to cellular hypoxia and trauma, causing further death of grafted dopamine neurons.[24] We recently demonstrated an increased dopamine neuron survival in transplants prepared from ventral mesencephalic (VM) tissue taken from transgenic mice that overexpress Cu/Zn superoxide dismutase (CuZn-SOD),[25] an enzyme which plays a critical role in the detoxification of oxygen free radicals.[14] This study strongly suggested that intracellular overexpression of CuZnSOD is an effective means of protecting dopamine neurons against free radicals generated during the grafting procedure.

§To whom correspondence should be addressed.

Abbreviations: Ad-hCuZnSOD, recombinant adenovirus encoding the human Cu/Zn SOD; Ad-RSVβgal, recombinant adenovirus encoding the *E. coli* β-galactosidase; βgal, β-galactosidase; CR3, complement receptor 3; DA, dopamine; HBSS, Hank's balanced salt solution; MOI, multiplicity of infection; 6-OHDA, 6-hydroxydopamine; PBS, phosphate-buffered saline; PD, Parkinson's disease; pfu, plaque forming unit; RSV, Rous sarcoma virus; SOD, superoxide dismutase; TH, tyrosine hydroxylase; VM, ventral mesencephalon.

One attractive strategy for producing high levels of bioactive proteins in neurons is the use of replication-defective adenovirus vectors, which allow delivery of transgenes into post-mitotic cells.[19] Thus, an adenovirus encoding CuZnSOD could be an interesting tool when trying to protect grafted dopamine neurons against free radical toxicity. We previously reported the construction of a replication-defective adenovirus containing human CuZnSOD cDNA (Ad-hCuZnSOD)[3]. This recombinant adenovirus efficiently drove the intracellular expression of human CuZnSOD *in vitro*, and was found to protect infected striatal cells in culture against glutamate neurotoxicity.

Reports regarding *ex vivo* or *in vivo* adenovirus-mediated gene transfer have recently focused on potentially detrimental effects of the gene transfer procedure. A marked inflammatory response has been described following *in vivo* injection into the rat striatum of a replication-defective adenovirus either containing or lacking the *E. coli* LacZ gene.[9] Moreover VM tissue infected *ex vivo* with adenoviral vectors encoding either *E. coli* LacZ or human brain-derived neurotrophic factor (BDNF) was reported to survive poorly upon subsequent intrastriatal grafting and presumably this was due to the increased host inflammatory response.[37] Therefore, the main objectives of the present study were to investigate further the feasability of the *ex vivo* adenovirus-mediated gene transfer approach and to examine whether it is possible to develop an effective gene transfer protocol that does not jeopardize graft survival or function.

Embryonic VM cells were genetically modified *ex vivo* to overexpress CuZnSOD using the hCuZnSOD recombinant adenovirus, and were subsequently transplanted into the denervated striatum of adult rats with unilateral 6-hydroxydopamine (6-OHDA) lesions of the mesostriatal pathway. We evaluated the survival and the functional efficacy of transplants infected with recombinant adenoviruses encoding either *E. coli* LacZ (Ad-RSVβgal) or human CuZnSOD (Ad-hCuZnSOD). In addition, the inflammatory response to cells subjected to adenoviral gene transfer was monitored by immunohistochemical detection of macrophages and microglia.

EXPERIMENTAL PROCEDURES

Recombinant adenoviral vectors

Recombinant replication-defective adenoviruses bearing the *Escherichia coli* LacZ marker gene (Ad-RSVβgal) or the human copper-zinc superoxide dismutase gene (Ad-hCuZnSOD) were obtained as described previously.[3,45] Briefly, the LacZ gene and the hCuZnSOD cDNA were inserted downstream from the long terminal repeat of the Rous Sarcoma Virus promoter (RSV) in a plasmid (shuttle vector) containing the inverted terminal repeat of the adenoviral genome, encapsidation sequences, and adenoviral sequences allowing homologous recombination with the right part of the viral genome. After their linearization, the shuttle vector and the large ClaI fragment of the type 5-adenovirus DNA were co-transfected into the transformed human kidney cell line 293 using the calcium phosphate-DNA precipitation method. The transfected cells were overlaid with agar, and plaques were screened for the presence of the recombinant adenovirus. Viral stocks were prepared by expansion and purification of the recombinant adenoviruses. Virus titres were determined by plaque assays on 293 cells and expressed as plaque forming units (pfu) /ml. Ad-hCuZnSOD and Ad-RSVβgal were obtained at titres of 3×10^{10} and 10^{11} pfu/ml, respectively.

Lesion surgery and turning behaviour

Unilateral lesion of the ascending mesostriatal dopamine pathway of adult female Sprague–Dawley rats (bred under Special Pathogen Free (SPF) conditions, B & K Universal, Sollentuna, Sweden) was achieved by stereotaxic injection of 6-OHDA (Sigma, Sweden) into the medial forebrain bundle as described previously.[15] The rats were tested for amphetamine-induced (2.5 mg/kg, i.p.) turning behaviour.[48] The rotational behaviour was monitored in automated rotameters for 90 min. A net rotational asymmetry score was calculated by subtracting the number of turns contralateral to the lesion from the number of ipsilateral turns. For transplantation surgery, we selected the rats that exhibited a net rotation score of at least six full turns/min towards the lesioned side, which is consistent with a near complete unilateral depletion of dopamine.[44] At two and five weeks after transplantation, the rats were tested again for rotational asymmetry using the same protocol.

Preparation and infection of embryonic ventral mesencephalon cells

Cell suspensions of VM tissue were obtained from embryonic day 14 rats derived from three pregnant Sprague–Dawley females. The embryonic brains were dissected in Hank's balanced salt solution (HBSS, Gibco, Sweden) and the pieces of VM tissue obtained were incubated in HBSS containing 0.1% trypsin (Worthington, USA)/0.05% DNase (Sigma, USA) at 37°C for 20 min. After repeated rinsing with HBSS/0.05% DNase, the tissue was mechanically dissociated into a single-cell suspension with fire-polished Pasteur pipettes. We prepared a total of three cell suspensions, each containing tissue from 12 embryos dissociated in 80 ml of medium. Two cell suspensions were then infected with either hCuZnSOD or RSVβgal recombinant adenoviruses. The viruses were directly added to the cell suspensions at a multiplicity of infection (MOI) of 25 pfu/cell, and the cell suspensions were then immediately incubated at 37°C for 1 h. Each cell suspension was agitated briefly every 15 min to promote mixing of the cells and viruses. A control cell suspension, mock-infected with buffer alone, was incubated under the same conditions. Cell concentration and viability was determined using Trypan Blue dye exclusion. For all three groups, the cell viability was over 90%, and the cell concentrations in the control, βgal and superoxide dismutase (SOD) groups were 3.65×10^4/μl, 4×10^4/μl and 3.99×10^4/μl, respectively.

Transplantation surgery

One or two months after having 6-OHDA lesion surgery, 24 rats were divided into three groups (n=8 each) and received control uninfected transplants (control group) or transplants infected with either Ad-RSVβgal (βgal group) or Ad-hCuZnSOD (SOD group). Two implants of cells (2 μl each, giving a total of 146,000–160,000 cells/rat) were stereotaxically deposited into the 6-OHDA denervated, right striatum of anaesthetized rats as described previously.[15] The injection coordinates were (with respect to bregma and dura) A: 0.7 mm, L: 2.3 mm and 3.2 mm, V: 4.5 mm, with the tooth bar set at zero.

Tissue preparation

To verify the transgene expression and to monitor the extent of inflammatory response, two grafted rats/group

were perfused four days after transplantation surgery. The remaining rats (n=6 for each group) were perfused five weeks after transplantation surgery. Rats were deeply anaesthetized with chloral hydrate and perfused transcardially with saline followed by a solution of 0.1 M phosphate buffer containing 4% paraformaldehyde, pH 7.4. Brains were removed, postfixed for 4 h in 4% paraformaldehyde, cryoprotected in 20% sucrose overnight, and 30 μm coronal sections were cut on a freezing microtome. Free-floating sections were processed for βgal histochemistry, and for tyrosine hydroxylase (TH), hCuZnSOD, βgal and OX-42 immunohistochemistry (for details see below).

β-galactosidase histochemistry

β-gal activity was detected by incubating the brain sections for 3 h at 37°C in an X-gal solution consisting of 0.1 M phosphate-buffered saline (PBS) with potassium ferricyanide (4 mM, Sigma), potassium ferrocyanide (4 mM, Merck, Germany), $MgCl_2$ (4 mM, Merck) and X-gal (0.4 mg/ml, Appligene, USA).

Immunohistochemistry

After quenching of endogenous peroxidase with 3% H_2O_2 in 0.1 M PBS, and blocking of nonspecific staining with 10% serum in 0.3% Triton X-100, brain sections were incubated overnight with primary antibodies against hCuZnSOD (Valbiotech, France, 1:500), β-gal (Cappel, USA, 1:1000), TH (Pel-Freez, USA, 1:500) or OX-42 (Serotec, UK, 1:300) at room temperature. Sections were then incubated with the following biotinylated secondary antibodies: anti-sheep/goat Ig (Amersham, UK), anti-rabbit Ig (Vector, USA), anti-rabbit Ig (Vector) and anti-mouse Ig (Vector) for hCuZnSOD, βgal, TH and OX-42, respectively. Labelling systems used were the streptavidin–biotinylated horseradish peroxidase complex (Amersham) for hCuZnSOD, and the avidin–biotin complex (Vectastain Elite kit, Vector) for the other proteins.

Double-labelling experiments were performed by processing the slides for βgal histochemistry, followed by TH- or OX42- immunohistochemistry.

Quantification of tyrosine hydroxylase-positive cells

The number of surviving TH-immunoreactive neurons in each graft was assessed by manual counting on every third section on blind-coded slides. The raw values were corrected according to the Abercrombie formula,[1] with the mean cell diameter estimated at 16.4 μm, 17.1 μm and 17.1 μm for the control, βgal and SOD groups, respectively.

Statistical analysis

A two-factor repeated measures analysis of variance (ANOVA) was used to determine differences in rotational asymmetry between groups over time. One-factor ANOVA with *post hoc* Scheffe's F-test was used for intergroup comparisons of rotation scores for single time-points, and paired t-test was used for intragroup comparisons over time. One factor ANOVA was used to compare numbers of surviving TH-immunoreactive neurons between groups.

RESULTS

Cell suspensions of embryonic VM tissue, either uninfected or infected with the βGal or SOD adenoviruses, were transplanted into the striatum of the denervated rats (eight rats/group). Two rats/group were perfused four days after transplantation for morphological analysis, and the other rats were tested for amphetamine-induced turning behaviour before sacrifice and histological examination.

Table 1. Amphetamine-induced rotation asymmetry

	Pregrafting	Two weeks postgrafting	Five weeks postgrafting
Control (n=6)	7.1±0.7	10.1±2.2	3.1±2.7
βgal (n=6)	9.9±2	9.2±2.6	4.7±2.1
SOD (n=6)	8.8±1.6	6.5±0.7	−6.3±1.7

Net rotational asymmetry score (full turns contralateral to the lesion substracted from turns ipsilateral to the lesion/min).
Data are given as means±SEM.

Rotational behaviour

The number of amphetamine-induced rotations ipsi- and contralateral to the lesion were monitored before transplantation, and two weeks and five weeks after transplantation. Net rotation asymmetry scores are summarized in Table 1. Prior to transplantation, the net ipsilateral rotation asymmetry was not different between the three groups (one factor ANOVA $F_{2,15}$=0.83, P>0.05). At two weeks after transplantation, the proportions of rats that exhibited at least 50% reduction in net motor asymmetry compared to pre-transplantation values were 0/6, 2/6, and 1/6, for the control, βgal and SOD groups, respectively. At five weeks post-transplantation, these proportions were 3/6, 4/6 and 6/6 (2/6 rats exhibited more contralateral turns than ipsilateral in each of the control or βgal groups, and this proportion was 5/6 for the SOD group). A two-factor repeated measures ANOVA revealed a significant difference for the net ipsilateral asymmetry between groups [$F_{2,45}$=5.44, P<0.01], within groups across time [$F_{2,45}$=18.1, P=0.0001], and between groups across time [group × time interaction, $F_{4,45}$=2.89, P<0.05]. At two weeks after implantation, the rotation score was not significantly different between groups [one-factor ANOVA $F_{2,15}$=0.88, P0.05]. At five weeks after surgery, there was a significant difference between groups for the net rotation asymmetry [one-factor ANOVA, $F_{2,15}$=7.54, P<0.01]: the rotation score was significantly reduced in the SOD group compared to the control and βgal groups (*post hoc* Scheffe's F-test, P<0.05 and P<0.01, respectively). Only the SOD group exhibited complete reversal of rotation asymmetry and displayed a mean score that was significantly reduced compared to pre-transplantation and two weeks post-transplantation values (paired Student's t-test, P<0.01).

Striatal expression of the transgenes

Human CuZnSOD cDNA and the *E. coli lacZ* gene were introduced into rat embryonic VM cells using the adenovirus infection method previously used for cell cultures and now modified for freshly-prepared cell suspensions. VM cells were infected at a MOI of 25 pfu/cell, a viral concentration that we have previously found to direct the production of

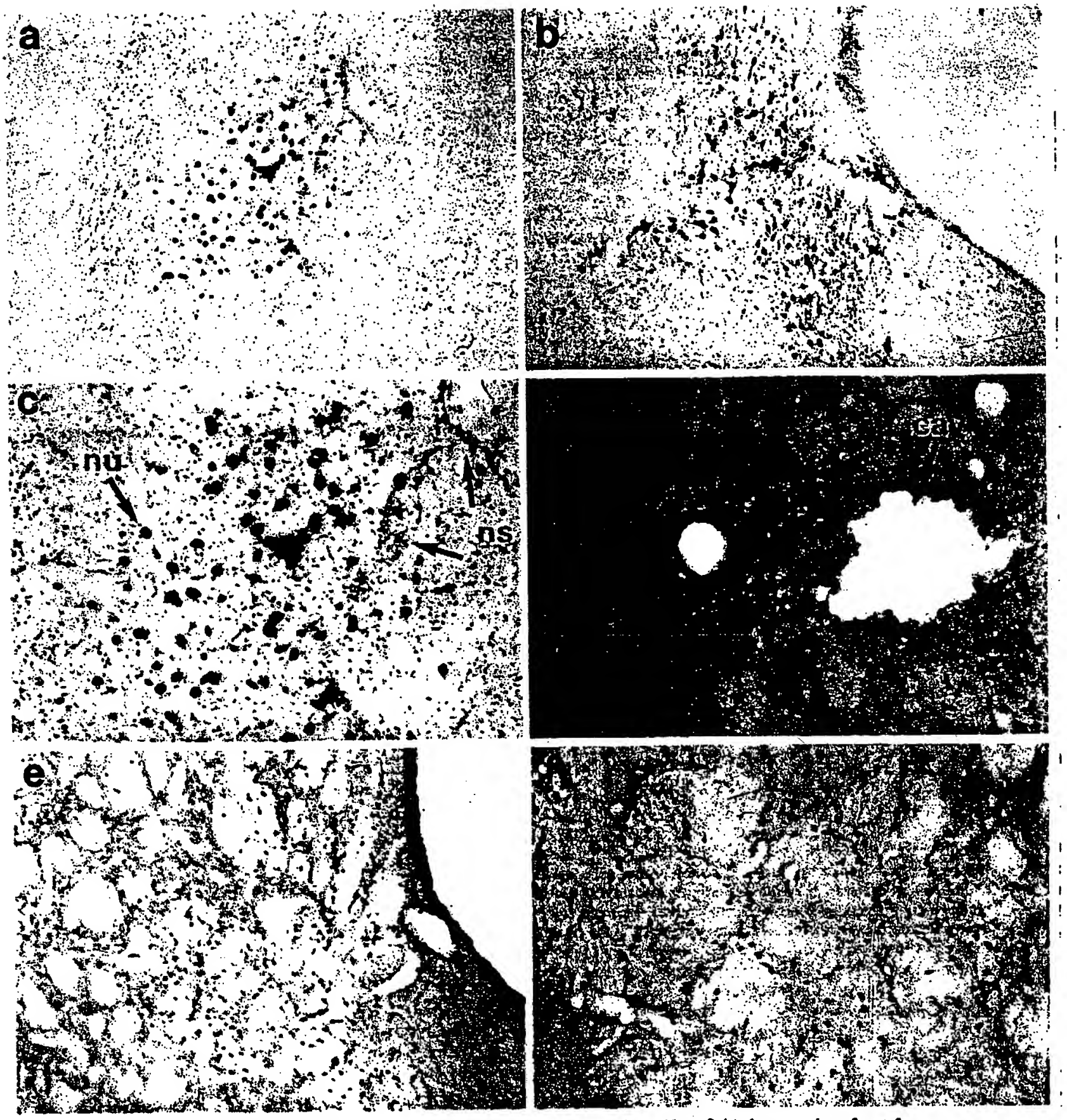


Fig. 1. X-gal histochemistry (a, b, c) and βgal immunohistochemistry (d, e, f) in the neural grafts at four days (a, c, d) and five weeks (b, e, f) after intra-striatal transplantation of embryonic mesencephalic tissue infected with the βgal adenovirus. a, b, d, e) low magnification of βgal-positive cells (scale bar=100 μm). An example of cavity (cav) is illustrated in d. c, f) high magnification of βgal-positive cells (scale bar=100 μm). Specific blue staining of βgal-positive nuclei (nu) and non-specific blue staining (ns) are illustrated in c.

hCuZnSOD or β-gal in cultured cells, without significant toxicity for TH-immunoreactive neurons (unpublished observations).

Expression of β-galactosidase. At four days after the graft surgery, two of the eight rats grafted with Ad-RSVβgal-infected cell suspensions were killed and showed surviving transplants (Fig. 1a,c,d). However, at this time-point, there were often cavities within the graft tissue in all groups (Fig. 1d). These cavities possibly represented areas where the graft tissue was not completely integrated into the host striatum. Using βgal-histochemistry (X-gal staining), several blue coloured nuclei were found to be

clustered inside the graft and around the injection site (approximately 50–150 βgal-positive cells/graft section, Fig. 1a,c). These nuclei correspond to βgal-producing cells in which the *lacZ* gene expression was targeted to the nucleus by the SV40 nuclear localization signal.[45]

The expression of βgal was still detected at five weeks after transplantation in the six transplanted rats of the βgal group using X-gal histochemistry (Fig. 1b) (approximately 50–150 βgal-positive cells/graft section).

With the X-gal staining procedure, it is possible to detect non-*E. coli* lacZ staining in cells with neuronal, glial and endothelial morphology in the normal adult rat brain;[35] we thus performed an immunohistochemical procedure using an antibody specific for the *E. coli* βgal. A similar labelling pattern was obtained whether using X-gal histochemistry (Fig. 1a,b,c) or βgal immunohistochemistry (Fig. 1d,e,f) staining procedures, although some non specific X-gal labelling was sometimes observed around blood vessels (Fig. 1c). No non-specific βgal immunostaining was observed in brain sections from rats grafted with Ad-hCuZnSOD-infected cell suspension (not shown).

As βgal was targeted to the nucleus, only nuclei were stained and this rendered the morphological characterization of cells impossible. Thus, we could not discriminate the cell types that were infected with the RSVβgal adenovirus. Co-labelling experiments were performed on a few sections using X-gal histochemistry and TH or OX-42 immunochemistry. There were several cells labelled with either antibody or the X-gal histochemistry; however, in these sections, we did not find any *lacZ*-positive cells that were clearly double-labelled.

Expression of human Cu/Zn superoxide dismutase. In rats implanted with Ad-hCuZnSOD-infected cell suspension, we assessed the expression of hCuZnSOD in the grafts using an antibody which discriminates between endogenous rodent CuZnSOD and the exogenous human form of the enzyme.[3] At four days after surgery, two of the eight rats grafted with the hCuZnSOD adenovirus were killed and these contained surviving transplants with human CuZnSOD-immunoreactive cells (Fig. 2a,c). As in the βgal group, cavities were found within the graft tissue. The immunoreactive cells were uniformly stained for the hCuZnSOD recombinant protein throughout the cytoplasm and dendritic processes, which permitted the identification of positive cells having neuron-like or glia-like morphology (Fig. 2c) (with approximately 20–50 hCuZnSOD-positive cells/graft section).

Human CuZnSOD immunostaining was still detected at five weeks post-implantation and morphological examination of the cells indicated that a minority of infected cells had a clear neuronal phenotype (Fig. 2b,d). Most of the cells, located inside or around the graft, resembled microglia (Fig. 2d). In most grafts, the intensity of the hCuZnSOD immunostaining appeared slightly weaker at five weeks postgrafting when compared to postgrafting at four days.

We did not detect any cortical X-gal or CuZnSOD labelling consecutive to retrogradely-transported free virus along the corticostriatal pathway in rats grafted with either Ad-hCuZnSOD- or Ad-RSVβgal-infected cell suspensions (not shown).

OX-42 immunohistochemistry

The presence of an inflammatory response was evaluated by OX-42 immunohistochemistry (Fig. 3). This antibody detects rat complement receptor type 3, which is located on macrophages and microglia.[34] At four days after transplantation, there was more intense OX-42 staining in the rats grafted with infected cell suspensions (Fig. 3b,c) than in the control group (Fig. 3a). No obvious difference was detected between rats grafted with either Ad-hCuZnSOD- or Ad-RSVβgal-infected transplants.

The majority of immunoreactive cells were found within the graft tissue and had an activated microglia phenotype with an enlarged intensely-stained soma and several distinctly-labelled processes with multiple arborizations. Several round or cuboidal cells without processes, which resembled macrophages, were also found within and around the grafts. In the contralateral hemisphere, there were several weakly-stained cells with the characteristics of resting microglia cells.

The intensity of OX-42 staining decreased significantly between four days and five weeks after transplantation in both the βgal (Fig. 3b,e) and SOD (Fig. 3c,f) groups. At five weeks postgrafting, most of the immunoreactive cells had a microglia morphology and were located at the interface between graft and host tissue.

Graft survival

Light microscopic analysis of TH-immunostained brain sections (Fig. 4) revealed two surviving grafts in the striatum of each rat from the SOD and the control groups. In one of the SOD rats, the two grafts were inadvertently placed in the caudal striatum, close to the globus pallidus. One of six control rats had two grafts that were located in the lateral striatum. In the βgal group, 3/6 rats had two surviving grafts, 2/6 had one surviving lateral graft, and 1/6 did not have any surviving graft (this later animal was thus excluded from the statistical analysis).

TH-immunoreactive neurons in each graft were added for each rat and the mean number of surviving TH-positive neurons in each group was 443 ± 129, 347 ± 130, and 700 ± 150 for the control, βgal and SOD group, respectively (Fig. 4). There was no statistically significant difference between groups for the mean number of surviving TH-positive cells in the

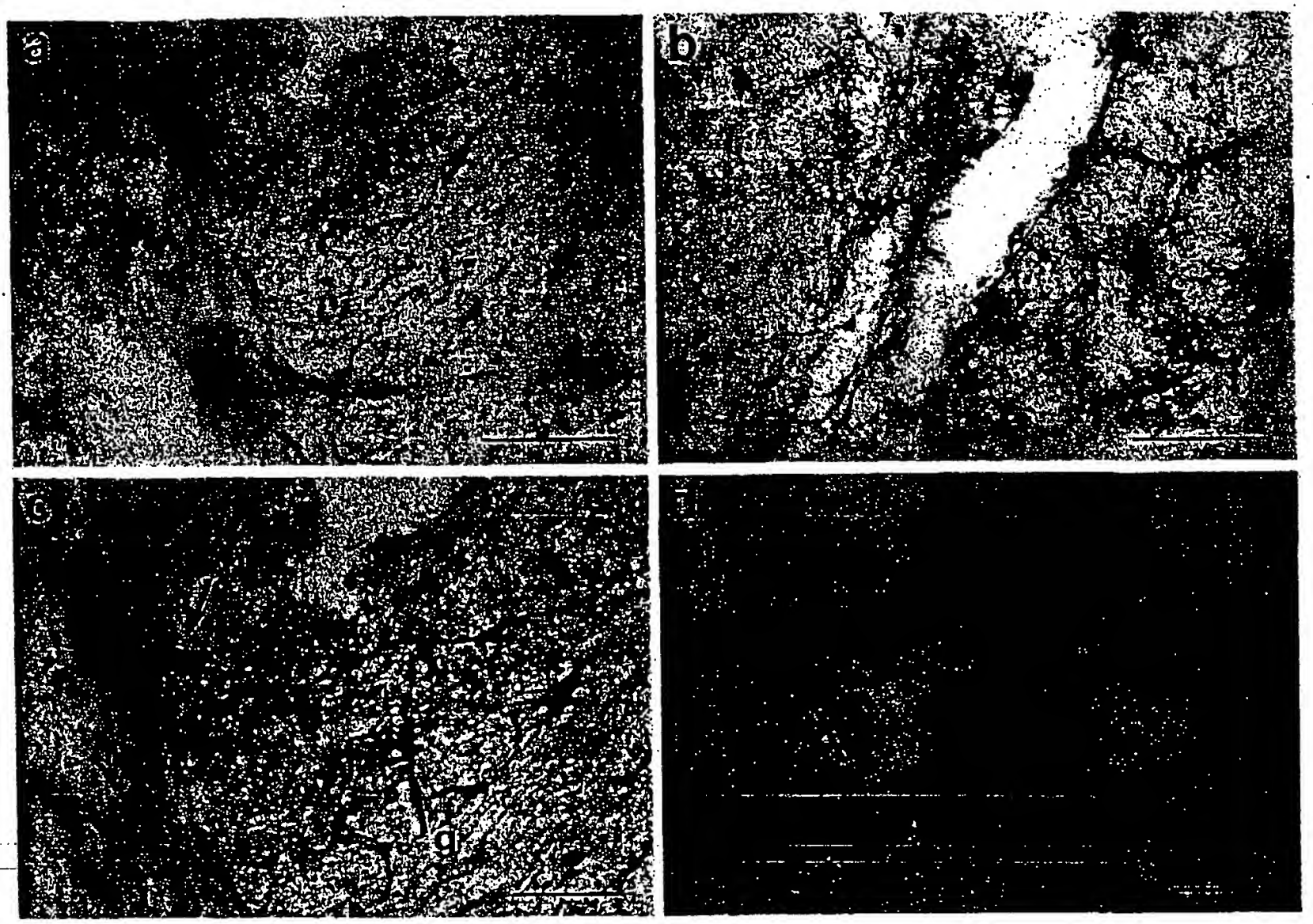


Fig. 2. Human CuZnSOD immunostaining of the neural grafts infected with the hCuZnSOD adenovirus analysed at four days (a, c) and five weeks (b, d) post-transplantation. a, b) Low magnification of hCuZnSOD-positive cells inside the graft (scale bar=100 μm). c) High magnification of hCuZnSOD-positive cells with a "neuron-like" (n) or "glia-like" (g) morphology (scale bar=30 μm). d) High magnification of hCuZnSOD-positive cells (scale bar=30 μm) showing a non-neuronal cell (arrow).

grafts (one-factor ANOVA, $P>0.05$), although a trend was observed with a higher TH cell number in the SOD group. Since approximately 160,000 cells were implanted in each animal, the mean yield of surviving TH-positive neurons in the three groups was approximately 3.1/1000 injected cells.

Correlation between graft survival and function

The sum of TH-positive cells found in each rat was plotted against the percent reduction in net rotation asymmetry at five weeks postgrafting. Logarithmic regression analysis showed a significant correlation between the two parameters ($r^2=0.510$, $P<0.01$). From the curve obtained by the analysis, we deduced that approximately 500 TH-positive neurons were necessary to induce a 50% recovery in rotational behaviour (unpublished observations).

DISCUSSION

Previous studies have shown that intrastriatal *in vivo* gene transfer using viral vectors encoding for the *TH* gene can result in significant behavioural recovery in 6-OHDA lesioned rats.[12,16,17] In these studies, viruses were directly injected into the denervated striatum. Thereby host striatal cells were genetically modified to produce dopamine (DA), most probably resulting in a reduction in striatal DA receptor supersensitivity which was manifested as an amelioration of apomorphine-induced motor asymmetry.[12,16,17] Alternatively, intrastriatal grafting of mesencephalic DA neurons can substitute for the lost DA innervation in the striatum of lesioned rats (for review see Ref. 4). Recent studies have demonstrated that in a rat model of PD, there was an improved survival of grafted dopamine neurons that had been treated with exogenous antioxidant[24] or taken from transgenic mice that overexpress hCuZnSOD.[25] Intracellular production of active hCuZnSOD protein in grafted neurons may be attained by using a replication-defective adenovirus vector for *ex vivo* transfer of the hCuZnSOD gene, i.e. *in vitro* gene transfer into cells that are subsequently transplanted into the brain. Such a method has been recently described for human neural progenitors infected *in vitro* by an adenovirus carrying the *E. coli lacZ* marker gene and then transplanted into the brain of

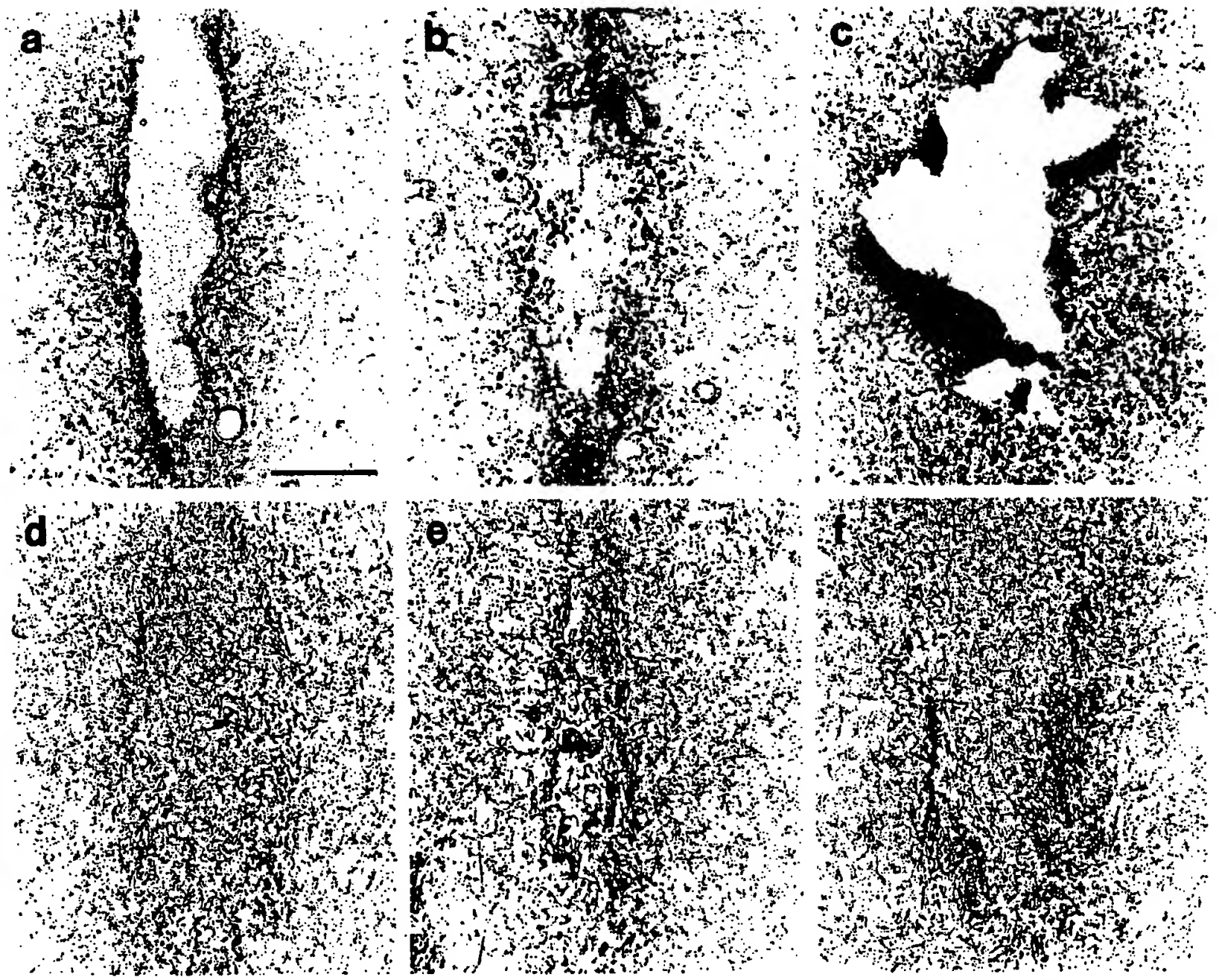


Fig. 3. Microglia/macrophage (CR3) immunostaining of coronal sections through the neural grafts at four days (a,b,c) and five weeks (d,e,f) after transplantation. a, d) Control; b, e) βgal; and c, f) SOD representative neural grafts (scale bar=250 μm).

adult rats.[38] The main objective of our present study was to examine whether embryonic VM tissue could be infected *in vitro* with an adenovirus and then grafted into the adult brain without a reduction in transplant survival or function. Our present results demonstrate that the hCuZnSOD and βgal proteins are expressed within and around intrastriatal grafts of embryonic dissociated mesencephalic tissue previously infected with adenoviruses bearing either the hCuZnSOD cDNA (Ad-CuZnSOD) or the *E. coli lacZ* gene (Ad-RSVβgal).

The adenovirus vector-driven expression of the exogenous proteins was seen at four days after transplantation and persisted for at least five weeks thereafter. Inflammatory consequences of the adenovirus gene transfer appeared minimal: only a moderate microglial response, detected using immunocytochemistry for complement receptor 3 (CR3), was observed around the graft tissue. This weak inflammatory response was probably induced by the virion particles rather than by the transgenes: direct intracerebral injection of replication-defective adenoviruses either containing or lacking the *E. coli lacZ* gene was previously reported to lead to a substantial inflammatory response mediated by the virion particles themselves.[9] At 30 days after injection, the authors reported that CR3 expression (labelling macrophages and microglia) did not differ between virus- and buffer-injected animals.[9] This is in agreement with our present observation of a moderate to intense CR3 expression in and around all grafts at four days after transplantation and a relatively low microglial response at five weeks after surgery. Furthermore, our results demontrate that adenoviral-infection of VM cell suspensions prior to intrastriatal implantation did not have detrimental effects on the survival of DA neurons in the graft. On the contrary, there was a trend for a better survival of TH-immunoreactive grafted cells derived from Ad-hCuZnSOD-infected cell suspensions. Our results differ from those in a recent study using a defective herpes simplex virus vector system to transfer *ex vivo*

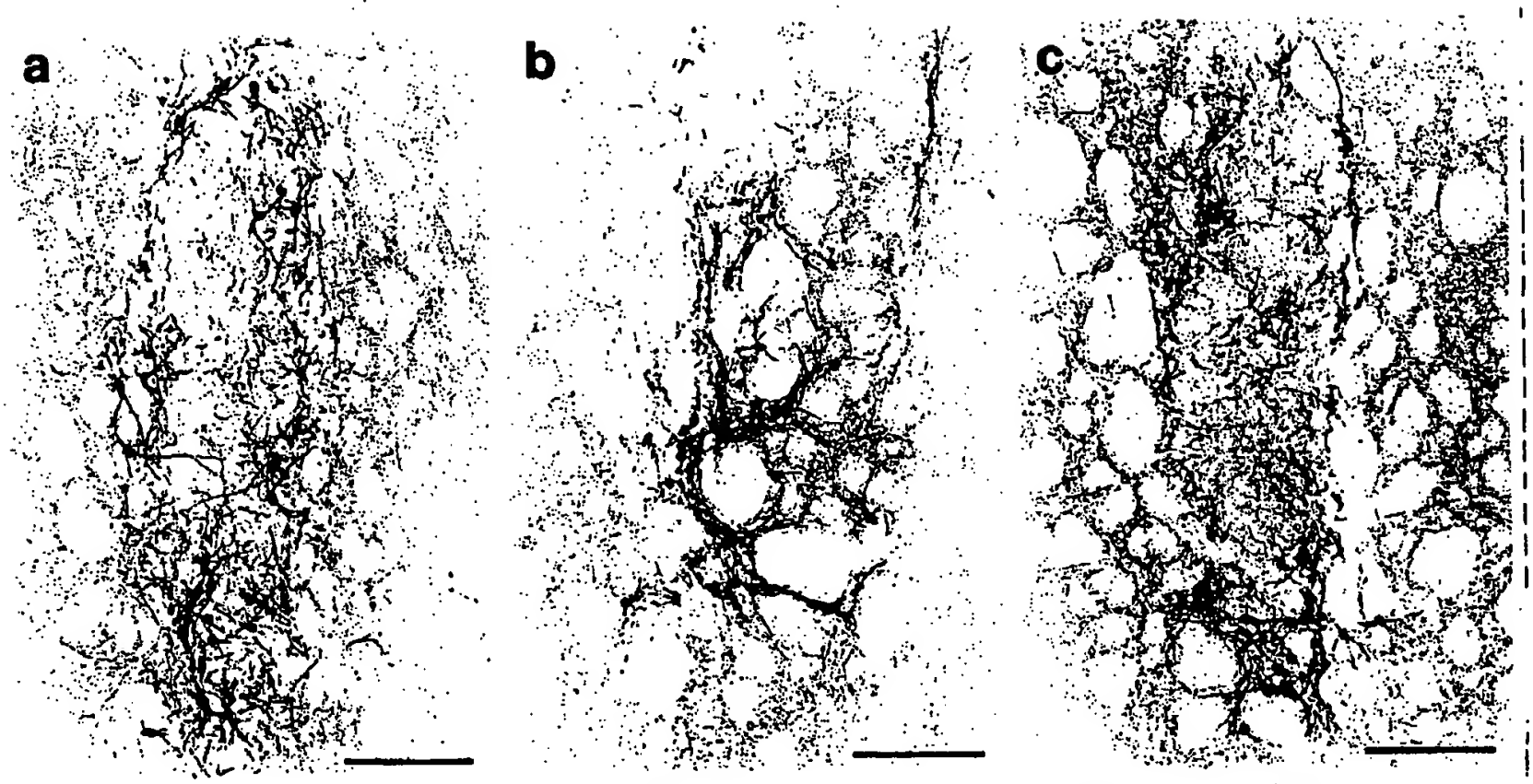


Fig. 4. TH-immunostaining of coronal sections through the neural grafts at five weeks after transplantation in one representative rat from each group. a) Control; b) βgal; and c) SOD groups are illustrated (scale bar=250 μm).

either the *lacZ* or the *TH* gene into the rat brain.[39] In this study, the authors infected neocortical cells prior to transplanting them into the rat striatum, but did not find a single TH-expressing cell in the grafts (they found only a few lacZ-positive cells). In another recent study using adenoviral vectors, addition of recombinant adenoviruses to mesencephalic tissue grafts reduced the survival and differentiation of TH-positive neurons in the transplants.[37] In this report, grafted cell suspensions had been supplied with 3×10^7 pfu of adenoviral vectors encoding either *E. coli* LacZ or human brain-derived neurotrophic factor. In our present study, the use of lower concentrations of recombinant adenovirus (4×10^6 total pfu) may explain why there was no impairment of graft survival and only a moderate inflammatory response in the transplants. Nevertheless, this low multiplicity of infection was sufficient to induce transgene expression in the brain grafts. Non-specific inflammation has been postulated to increase the risk of rejection of histoincompatible neural grafts by up-regulation of transplantation antigens.[49] This mechanism is not applicable to our present experiment, because the donor-host combination was syngeneic. Conceivably, expression of viral antigens on the surface of grafted cells may stimulate immune destruction of some infected cells.[2] However, our results suggest that there was no apparent intense immune rejection of adenovirus-infected cells.

The use of Ad-hCuZnSOD and Ad-RSVβgal for *ex vivo* gene transfer did not seem to inhibit the functional capacity of the grafted cells and their ability to reverse motor asymmetry in rats with experimental PD. In fact, the onset of functional transplant effects in rats transplanted with Ad-hCuZnSOD-infected cell suspensions was faster than in those receiving untreated control tissue or grafts infected with Ad-RSVβgal. The significantly faster and more extensive functional recovery observed in the SOD group was not reflected in a statistically significant increase in DA graft cell number in this group, although there was a strong trend for enhanced graft survival. The number of surviving DA neurons per grafted embryonic VM (each host received the equivalent of 0.6 embryonic VM) was 700–1200, which is in the lower range of previously reported results.[22,24,26,43] The relatively poor survival rate in all groups may be related to the fact that in this experiment the tissue was vigorously dissociated into single-cell suspensions, rather than maintained as a mixture of single cells, and small aggregates.[7] There is strong evidence from several earlier studies[6,24,25,41,43] that the number of surviving DA neurons in a graft governs the extent of recovery in the amphetamine-induced rotation test. In our present study, there was also a significant correlation between the logarithm of the number of TH-positive cells and the percentage reduction in rotation asymmetry. It may thus seem surprising that the SOD group, which displayed greater functional recovery, did not carry significantly larger grafts. However, recovery of rotational behaviour is also related to precise graft placement[11] and extent of fibre outgrowth.[5] Small variations in these parameters, combined with the strong trend towards larger grafts could have resulted in the observed enhanced functional effects.

The trend for an improved graft survival in the SOD group, compared to the βgal and control group, could be associated with the potential neuroprotective effect of intracellular overexpression of SOD. This enzyme plays a crucial role in the free radical detoxification by scavenging the superoxide anion (O_2^-) which has been shown to be neurotoxic either directly or through its reaction with nitric oxide.[10,18,47] Oxidative stress potentially generated by cellular hypoxia and trauma that occur during preparation and implantation of embryonic mesencephalic tissue could therefore be reduced by intracellular overexpression of SOD in grafted neurons. Possibly, SOD expressed in non-neuronal cells may also indirectly enhance the functional recovery of the grafts by increasing the survival and/or function of astrocytes stimulated in the graft or in the host tissue: glial cells are known to play an important role in the survival of neurons by secreting growth factors (such as glial-derived neurotrophic factor[20]) or by providing cysteine which is taken up by neurons for glutathione synthesis.[40] Reactive oxygen species are potentially generated by activated phagocytes (e.g., brain macrophages and microglia):[23] these possess an enzymatic complex, the NADPH oxidase, which catalyses the formation of superoxide upon exposure of phagocytes to appropriate stimuli (infection, grafting,..). A defective phagocyte superoxide production has been reported to alter the inflammatory response in a mouse model of chronic granulomatous disease.[32] In rats grafted with the Ad-hCuZnSOD-infected cell suspensions, the over-expression of SOD in infected phagocytes may lead to defective immune response mechanisms linked to superoxide generation. This could explain the tendency of an enhanced graft survival and the significant behavioural recovery in the SOD group. It is well known that free radical species can diffuse over membranes and that by-products of lipid hydroperoxides can damage the membranes of neighbouring cells[30] and quenching of these by non-neuronal cells could also be relevant for protection of the DA cells (by disruption of chain reactions of peroxidation).

In our study, all grafted rats had surviving transplants, and if infected, all of the transplants expressed the βgal or hCuZnSOD transgenes. A differential expression of the hCuZnSOD and βgal proteins was found in the grafts (number of βgal-immunoreactive cells hCuZnSOD-immunoreactive cells), which may be due to possible differences in the sensitivity of the two immunostaining procedures, or the stability of the two proteins.

The number of the *ex vivo* infected cells that expressed the exogenous proteins appeared lower in our study than in the report described by Sabaté *et al.*[38] However, this may be explained by one or more of the differences in the experimental protocols between the two studies. For example, ten times fewer cells were injected in our present study (150,000 cells/rat instead of 1,000,000), a lower viral concentration was employed (25 pfu instead of 500 pfu), the incubation time in the viral solution was reduced (1 h instead of overnight) and we performed the *ex vivo* infection in a cell suspension instead of in culture. The changes in most of these experimental conditions were necessary to optimize conditions for the survival of the DA neurons.

At four days after the graft surgery, cells expressing the transgenes had a neuronal and/or a glial phenotype. However, at five weeks post-transplantation, most transgene-expressing cells resembled microglia, although sporadic cells with neuronal morphology were found in the grafts. That may reflect a higher down-regulation of the transgene expression in neurons than in microglia, or differences in sensitivity of neurons and microglia to the immune reaction triggered by the viral infection. Unlike at four days postgrafting, many labelled cells were found out of the grafts at five weeks postgrafting (Fig. 1b,e), which may correspond to their migration away from the implantation site to populate a larger striatal area.[27]

Double-labelling experiments failed to show a co-localization of the βgal transgene and the TH. However, previous *in vitro* experiments showed that approximately 5–10% of the TH-infected VM cells were co-labelled with βgal (data not shown). *In vivo* experiments of direct Ad-RSVβGal injection into the substantia nigra also previously showed that about 50% of βgal-positive cells in the substantia nigra co-expressed TH, thereby demonstrating the potentiality of DAergic neurons to be infected with adenoviruses.[19] As few TH cells were found in the sections that we used for the double-labelling experiment (10–20 TH-positive cells), it is not surprising that none of them were clearly βgal-positive. Moreover, our double-staining analysis was carried out at five weeks after transplantation, and with regard to not finding double-labelled TH cells at this time-point does not exclude the possibility that such TH cells expressed the transgenes at the moment of the grafts. Previous *in vitro* experiments where the embryonic mesencephalic rat cells were infected with the SOD adenovirus provided double-immunostaining evidence that at least 5–10% of the TH cells clearly expressed exogenous hCuZnSOD a few days after infection (unpublished observations). Oxidative stress is potentially generated by cellular hypoxia and trauma that occur during preparation and implantation of embryonic tissue and could have deleterious effects only during the first hours and days after transplantation. In this context, only the early expression of SOD within the cells should be essential for neuroprotection. Furthermore, a down-regulation of transgene expression has already been observed in transplanted cells genetically modified using retroviruses, and could be a result of gene regulation when using viral promoters.[31] In our present study, such a suppression of the transgene expression (under the control of the RSV promoter)

could explain the failure to find clearly double-labelled TH neurons at five weeks postgrafting.

Possibly, host striatal cells could be infected by free adenoviruses remaining in the supernatant of the grafted cell suspensions. However, after 1 h incubation at 37°C, most of the free virions should have been inactivated. If there had been free virions present, probably some of them would have been taken up by nerve terminals at the injection site and transported retrogradely[9,13,33] e.g., along the corticostriatal pathway. Thus, the absence of cells exhibiting labelling for βgal or CuZnSOD in the cortex of the rats in our present study suggests that there were not significant amounts of free virions in the cell suspension supernatants.

CONCLUSION

Here, we described a promising *ex vivo* gene transfer method using a replication-defective adenovirus bearing the human CuZnSOD gene, which could be used in attempts to enhance functional efficacy and maybe survival of grafted DA neurons. Further studies will be essentially directed towards the improvement of the infection efficiency, to obtain a high level transgene expression with a minimal inflammatory reaction.

Acknowledgements—We thank M. C. Geoffroy, I. Loquet and J. J. Robert for their contribution to preparation of adenoviruses, and gratefully acknowledge the expert technical assistance of Britt Lindberg. We also thank P. Horellou and O. Sabaté for critically reading the manuscript and for their useful advice. This work was supported by the Centre National de la Recherche Scientifique, the Institut National de la Santé et de la Recherche Médicale, Rhône-Poulenc Rorer, the Institut pour la Recherche sur la Moelle Epinière, the Human Science Frontier program, the Swedish Medical Research Council (B96-12XB-11286-02B), the Crafoord, Kock and NHR Foundations, the Swedish Society of Medicine, the Swedish Society for Medical Research and the Medical Faculty of Lund. M.B. was supported by a Biotechnology program of the European Commission.

REFERENCES

1. Abercrombie M. (1946) Estimation of nuclear population from microtome section. *Anat. Rec.* 94, 239–247.
2. Allan J. E. and Doherty P. C. (1985) Immune T cells can protect or induce fatal neurological disease in murine lymphocytic choriomeningitis. *Cell Immunol.* 90, 401–407.
3. Barkats M., Bemelmans A.-P., Geoffroy M.-C., Robert J.-J., Horellou P., Revah F. and Mallet J. (1996) An adenovirus encoding CuZnSOD protects cultured striatal neurons against glutamate toxicity. *NeuroReport* 7, 497–501.
4. Björklund A. (1992) Dopaminergic transplants in experimental Parkinsonism: cellular mechanisms of graft-induced functional recovery. *Curr. Opin. Neurobiol.* 2, 683–689.
5. Björklund A. and Dunnett S. B. (1980) Reinnervation of the denervated striatum by substantia nigra transplants: functional consequences as revealed by pharmacological and sensorimotor testing. *Brain Res.* 20, 307–333.
6. Brundin P., Barbin G., Strecker R. E., Isacson O., Prochiantz A. and Björklund A. (1988) Survival and function of dissociated rat dopamine neurones grafted at different developmental stages or after being cultured *in vitro*. *Devl Brain Res.* 39, 233–243.
7. Brundin P. and Sauer H. (1991) Grafting human fetal brain tissue: a practical guide. In *Intracerebral Transplantation in Movement Disorders* (eds Lindvall O., Björklund A. and Widner H.), pp. 171-182. Elsevier, New York.
8. Brundin P., Strecker R. E., Widner H., Clarke D. J., Nilsson O. G., Astedt B., Lindvall O. and Björklund A. (1988) Human fetal dopamine neurons grafted in a rat model of Parkinson's disease: immunological aspects, spontaneous and drug-induced behaviour, and dopamine release. *Expl Brain Res.* 70, 192–208.
9. Byrnes A. P., Rusby J. E., Wood M. J. A. and Charlton H. M. (1995) Adenovirus gene transfer causes inflammation in the brain. *Neuroscience* 66, 1015–1024.
10. Dawson V. L. and Dawson T. M. (1996) Free radicals and neuronal cell death. *Cell death different.* 3, 71–78.
11. Dunnett S. B., Björklund A., Schmidt R. H., Stenevi U. and Iversen S. D. (1983) Intracerebral grafting of neuronal cell suspensions. IV. Behavioural recovery in rats with unilateral 6-OHDA lesions following implantation of nigral cell suspensions in different brain sites. *Acta physiol. scand.* 522, 29–37.
12. During M. J., Naegele J. R., O'Malley K. L. and Geller A. I. (1994) Long-term behavioral recovery in parkinsonian rats by an HSV vector expressing tyrosine hydroxylase. *Science* 266, 1399–1403.
13. Finiels F., Gimenez y Ribotta M., Barkats M., Samolyk M.-L., Robert J.-J., Privat A., Revah F. and Mallet J. (1996) Specific and efficient gene transfer strategy offers new potentialities for the treatment of motor neurone disease. *NeuroReport* 7, 373–378.
14. Fridovich I. (1995) Superoxide radical and superoxide dismutases. *A. Rev. Biochem.* 64, 97-112.
15. Frodl E., Duan W.-M., Sauer H., Kupsch A. and Brundin P. (1994) Human embryonic dopamine neurons xenografted to the rat: effects of cryoconservation and varying regional source of donor cells on transplant survival, morphology and function. *Brain Res.* 647, 286–298.
16. Horellou P., Vigne E., Castel M.-N., Barnéoud P., Colin P., Perricaudet M., Delaère P. and Mallet J. (1994) Direct intracerebral gene transfer of an adenoviral vector expressing tyrosine hydroxylase in a rat model of Parkinson's disease. *NeuroReport* 6, 49–53.
17. Kaplitt M. G., Leone P., Samulski R. J., Xiao X., Pfaff D. W., O'Malley K. L. and During M. (1994) Long-term expression and phenotypic correction using adeno-associated virus vectors in the mammalian brain. *Nature Genet.* 8, 148–153.
18. Lafon-Cazal M., Pietri S., Culcasi M. and Bockaert J. (1993) NMDA-dependent superoxide production and neurotoxicity. *Nature* 364, 535–537.
19. Le Gal La Salle G., Robert J.-J., Berrard S., Ridoux V., Stratford-Perricaudet L. D., Perricaudet M. and Mallet J. (1993) An adenovirus vector for gene transfer into neurons and glia in the brain. *Science* 259, 988–990.
20. Lin l
facto
21. Lind
S. B.
22. May
ventr
23. Mor
mole
24. Nak
embr
25. Nak
Cu/Z
Med
26. Nikk
appr
63, 5
27. Oko
adul
28. Olan
29. Olan
disea
30. Paci
theo
31. Palm
after
32. Poll
Dina
supe
33. Ridc
func
34. Rob
mon
57, 2
35. Rose
E. c
36. Rose
survi
37. Roy
mese
imm
38. Saba
to th
256–
39. Sabe
herp
40. Saga
1672
41. Saue
graft
42. Saue
func
43. Saue
func
44. Schn
susp
syntl
45. Strat
skele
46. Taka
incre
47. Troy
supe
48. Ung
6-hy
49. Widn
revie
50. Yure
by g

20. Lin L.-F. H., Doherty D. H., Lile J. D., Bektesh S. and Collins F. (1993) GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. *Science* **260**, 1130–1132.
21. Lindvall O. (1994) Neural transplantation in Parkinson's disease. In *Functional Neural Transplantation* (eds Dunnett S. B. and Björklund A.), pp. 103-137. Raven Press, New York.
22. Mayer E., Fawcett J. W. and Dunnett S. B. (1993) Basic fibroblast growth factor promotes the survival of embryonic ventral mesencephalic dopaminergic neurons-II. Effects on nigral transplants *in vivo*. *Neuroscience* **56**, 389–398.
23. Morel F., Doussiere J. and Vignais P. V. (1991) The superoxide-generating oxidase of phagocytic cells. Physiological, molecular and pathological aspects. *Eur. J. Biochem.* **201**, 523–546.
24. Nakao N., Frodl E. M., Duan W.-M., Widner H. and Brundin P. (1994) Lazaroids improve the survival of grafted rat embryonic dopamine neurons. *Proc. natn. Acad. Sci. U.S.A.* **91**, 12408–12412.
25. Nakao N., Frodl E. M., Widner H., Carlson E., Eggerding F. A., Epstein C. J. and Brundin P. (1995) Overexpressing Cu/Zn superoxide dismutase enhances survival of transplanted neurons in a rat model of Parkinson's disease. *Nature Med.* **1**, 226–231.
26. Nikkah G., Olsson M., Eberhard J., Bentlage C., Cunningham M. and Björklund A. (1994) A microtransplantation approach for cell suspension grafting in the rat Parkinson model: a detailed account of the methodology. *Neuroscience* **63**, 57–72.
27. Okoye G. S., Powell E. M. and Geller H. M. (1995) Migration of A7 immortalized astrocytic cells grafted into the adult rat striatum. *J. comp. Neurol.* **362**, 524–534.
28. Olanow C. W. (1992) An introduction to the free radical hypothesis in Parkinson's disease. *Ann. Neurol.* **32**, S2–S9.
29. Olanow C. W., Kordower J. H. and Freeman T. B. (1996) Fetal nigral transplantation as a therapy for Parkinson's disease. *Trends Neurosci.* **19**, 102–109.
30. Pacifici R. E. and Davies K. J. A. (1991) Protein, lipid and DNA repair systems in oxidative stress: the free radical theory of aging revisited. *Gerontology* **37**, 36–180.
31. Palmer T. D., Rosman G. J., Osborne W. R. A. and Miller D. (1991) Genetically modified skin fibroblasts persist long after transplantation but gradually inactivate introduced genes. *Proc. natn. Acad. Sci. U.S.A.* **88**, 1330–1334.
32. Pollock J. D., Williams D. A., Gifford M. A., Li L. L., Du X., Fisherman J., Orkin S. H., Doerschuk C. M. and Dinauer M. C. (1995) Mouse model of X-linked chronic granulomatous disease, an inherited defect in phagocyte superoxide production. *Nature Genet.* **9**, 202–209.
33. Ridoux V., Robert J.-J., Zhang X., Perricaudet M., Mallet J. and Le Gal La Salle G. (1994) Adenoviral vectors as functional retrograde neuronal tracers. *Brain Res.* **648**, 171–175.
34. Robinson A. P., White T. M. and Mason D. W. (1986) Macrophage heterogeneity in the rat as delineated by two monoclonal antibodies MRC 0X-41 and MRC OX-42, the latter recognizing complement receptor type 3. *Immunology* **57**, 239–247.
35. Rosenberg W. S., Breakefield X. O., De Antonio C. and Isaacson O. (1992) Authentic and artifactual detection of the *E. coli lacZ* gene product in the rat brain by histochemical methods. *Molec. Brain Res.* **16**, 311–315.
36. Rosenblad C. and Martinez-Serrano A. and Björklund A. (1996) Glial cell-line derived neurotrophic factor increases survival, growth and function of intrastriatal fetal nigral dopaminergic grafts. *Neuroscience*. (In press).
37. Roy E., Grégoire F., Tardif P., Bédard J. and Gravel C. (1995) Addition of adenoviral vectors to embryonic ventral mesencephalon cell suspensions used for striatal implantation into 6-OHDA-denervated rats induces detrimental immune responses. *Soc. Neurosci. Abstr.* **21**, 1980.
38. Sabaté O., Horellou P., Vigne E., Colin P., Perricaudet M., Buc-Caron M.-H. and Mallet J. (1995) Transplantation to the rat brain of human neural progenitors that were genetically modified using adenoviruses. *Nature Genet.* **9**, 256–260.
39. Sabel B. A., Vick A. and Hollt V. (1995) Neurotoxic reactions of CNS cells following gene transfer with defective herpes simplex virus (HSV-1) vector. *NeuroReport* **6**, 2447–2449.
40. Sagara J. I., Miura K. and Bannai S. (1993) Maintenance of neuronal glutathione by glial cells. *J. Neurochem.* **61**, 1672–1676.
41. Sauer H. and Brundin P. (1991) Effects of cool storage on survival and function of intrastriatal ventral mesencephalic grafts. *Restor. Neurol. Neurosci.* **2**, 123–135.
42. Sauer H., Fischer W., Nikkhah G., Wiegand S. J., Brundin P., Lindsay R. and Björklund A. (1993) BDNF enhances function rather than survival of intrastriatal dopamine cell-rich grafts. *Brain Res.* **626**, 37–44.
43. Sauer H., Frodl E. M., Kupsch A., Ten Bruggencate G. and Oertel W. H. (1992) Cryopreservation, survival and function of intrastriatal fetal mesencephalic grafts in a rat model of Parkinson's disease. *Expl Brain Res.* **90**, 54–62.
44. Schmidt R. H., Björklund A., Stenevi U., Dunnett S. B. and Gage F. H. (1983) Intracerebral grafting of neuronal cell suspensions. III. Activity of intrastriatal nigral suspension implants as assessed by measurements of dopamine synthesis and metabolism. *Acta physiol. scand.* **522**, 19–28.
45. Stratford-Perricaudet L. D., Makeh I. and Perricaudet M. (1992) Widespread long-term gene transfer to mouse skeletal muscles and heart. *J. clin. Invest.* **90**, 626–630.
46. Takayama H., Ray J., Raymon H. K., Hogg J., Fisher L. J. and Gage F. H. (1995) Basic fibroblast growth factor increases dopaminergic graft survival and function in a rat model of Parkinson's disease. *Nature Med.* **1**, 53–58.
47. Troy C. M., Derossi D., Prochiantz A., Greene L. A. and Shelanski M. L. (1996) Down-regulation of Cu/Zn superoxide dismutase leads to cell death via the nitric oxide-peroxynitrite pathway. *J. Neurosci.* **16**, 253–261.
48. Ungerstedt U. and Arbuthnott G. W. (1970) Quantitative recording of rotational behavior in rats after 6-hydroxydopamine lesions of the nigrostriatal dopamine system. *Brain Res.* **24**, 486–493.
49. Widner H. and Brundin P. (1988) Immunological aspects of grafting in the mammalian central nervous system. A review and speculative synthesis. *Brain Res. Rev.* **13**, 287–324.
50. Yurek D. M., Lu W., Hipkens S. and Wiegand J. (1996) BDNF enhances the functional reinnervation of the striatum by grafted fetal dopamine neurons. *Expl Neurol.* **137**, 105–113.

(*Accepted* 26 *September* 1996)

CURRENT AWARENESS TITLE

BRAIN RESEARCH

INTERNATIONAL MULTIDISCIPLINARY JOURNAL DEVOTED TO FUNDAMENTAL RESEARCH IN THE BRAIN SCIENCES

EDITOR-IN-CHIEF—D.P. PURPURA

ELSEVIER

VOL. 647 NO. 2 | Published weekly | 6 JUNE 1994

Completing Volume 647

ISSN 0006-8993
BRREAP 647 (2) 181–360 (1994)

ELSEVIER

BRAIN RESEARCH

Brain Research 647 (1994) 286–298

Research Report

Human embryonic dopamine neurons xenografted to the rat: effects of cryopreservation and varying regional source of donor cells on transplant survival, morphology and function

Eva M. Frodl [a,b], Wei-Ming Duan [a], Hansjörg Sauer [a,b], Andreas Kupsch [b], Patrik Brundin [a,*]

[a] *Restorative Neurology Unit, Department of Neurology, University Hospital, S-221 85 Lund, Sweden*

[b] *Department of Neurology, Klinikum Grosshadern and Department of Physiology, Ludwig-Maximilians-Universität, Munich, Germany*

Accepted 22 February 1994

Abstract

When grafting human mesencephalic tissue to patients suffering from Parkinson's disease, the number of surviving dopamine (DA) neurons in the graft is probably crucial. It may be possible to increase the number of DA neurons available for grafting to a patient by pooling tissue from many human embryos collected over several days or by obtaining more DA neurons from each embryo. We have addressed these issues by cryopreserving human mesencephalic DA neurons prior to transplantation and also by grafting human embryonic diencephalic DA neurons. The effects of cryopreservation were assessed 4–15 weeks after xenografting ventral mesencephalic tissue into the DA-depleted striatum of immunosuppressed rats with unilateral 6-hydroxydopamine lesions of the mesostriatal pathway. Control rats grafted with fresh mesencephalic tissue displayed robust reductions in amphetamine-induced turning following transplantation. Functional effects of the cryopreserved mesencephalic grafts were only observed in the one rat out of nine which contained the largest graft in this group. The number of tyrosine hydroxylase immunoreactive neurons in animals transplanted with cryopreserved tissue was significantly reduced to 9% of fresh tissue control grafts. Morphological analysis showed that cryopreserved DA neurons were approximately 22% and 28% smaller regarding the length of the long and short axis, respectively, when compared to the neurons found in fresh grafts. In the second part of the study, the survival and function of human embryonic diencephalic DA neurons were examined following xenografting into the DA-depleted rat striatum. A reduction of motor asymmetry was observed in two out of seven diencephalon-grafted rats. This finding was consistent with a good graft survival in these particular rats, which both contained large grafts rich in tyrosine hydroxylase immunoreactive neurons. Moreover, there was immunopositive staining for graft-derived fibers in the rat striatum containing tyrosine hydroxylase and human neurofilament, both in rats grafted with mesencephalic and diencephalic DA neurons. These findings suggest that cryopreservation, using the current technique, is not a suitable storage method for use in clinical trials of DA neuron grafting in Parkinson's disease. On the other hand, the application of alternative sources of DA neurons may in the future develop into a strategy which can increase the number of neurons obtainable from each human embryo.

Key words: Neural transplantation; Parkinson's disease; Dopamine; Cryopreservation; Diencephalon; Mesencephalon; Human embryo

1. Introduction

Neural transplantation has developed into a feasible therapeutic strategy for neurodegenerative diseases, in particular Parkinson's disease (PD) [17,18,27–29,49].

In accordance with animal experiments [5,6,8,39], the beneficial functional effects are believed to depend on a critical number of grafted dopamine (DA) neurons surviving in the patient's brain. However, in rat experiments [39] it has been estimated that only 5–20% of the dissected DA neurons survive the transplantation procedure and obtaining grafts that contain sufficient numbers of DA neurons in humans can pose a problem. A straightforward strategy to achieve grafts

* Corresponding author. Fax: (46) (46) 188150

SSDI 0006-8993(94)00261-A

containing DA neurons that exceed the crucial threshold number is to pool ventral mesencephalic (VM) tissue derived from several donor embryos. Thus, the implantation of tissue from 3 to 4 human embryos has led to significant clinical improvement and a markedly increased signal in fluorodopa positron emission tomography scans [27].

This need to harvest tissue from several embryos for each patient poses a practical problem in that surgery can only be conducted on days when sufficient amounts of donor tissue are available. Two possible solutions to this problem are: (1) to develop a long-term storage method for neural tissue which permits collection of donor material over several weeks without major loss of mesencephalic DA neuron viability; (2) to enhance the number of DA cells harvested from each embryo, not necessarily by increasing neuronal graft survival but by seeking additional groups of dopaminergic neurons in the embryonic brain.

To address the issue of long-term storage, in the present study, DA neurons were frozen to a temperature of −196°C and stored for 3 or 8 days before thawing and transplantation. The survival and functional capacity of the cryopreserved neurons was compared to that of identical cells obtained from the same embryos and grafted without a prior freezing period (non-frozen). Previous work has shown that it is possible to store mesencephalic DA neurons for 3–5 days at 4°C without major loss of graft viability, however, longer storage periods lead to impaired graft survival [19,39]. Freeze storage is an interesting alternative in that the length of the storage period does not seem to be a crucial parameter for neuronal survival following thawing [12,13,43,46]. We have previously demonstrated that approximately 40% of frozen, compared to non-frozen, rat embryonic DA neurons survive grafting to the rat striatum [40].

In an attempt to increase the total number of DA neurons obtainable from a single human embryo, we dissected out and grafted the diencephalic primordia of human embryos [30]. Using amphetamine-induced rotational behaviour, tyrosine hydroxylase (TH)- and human neurofilament (HNF)-immunocytochemistry, we investigated whether these neuronal populations survive and exert functional effects when implanted into the DA-depleted rat striatum.

2. Materials and methods

2.1. Experimental design

Human embryonic mesencephalic and diencephalic tissue were obtained from routine induced abortions (with the permission of the ethical committee at the Medical Faculty of the University of Lund), at ages ranging between 6 and 7.5 weeks post-conception (crown-to-rump length 17–28 mm). Tissue from a total of 6 embryos was used in grafting experiments and the brain of one embryo was used for morphological analysis.

2.1.1. Experiment 1

Human mesencephalic tissue assigned to experiment (Expt.) 1 was collected from 3 embryos and stored at 4°C in hibernation medium [26,39] for 24 h either prior to transplantation (HIB; $n = 8$) or prior to 3 days of freeze storage before grafting (HIB-F); $n = 5$). Grafted animals were left to survive for 30 to 35 days and their grafts were then quantitatively assessed for DA neuron survival in TH-immunostained sections. These animals were not tested for amphetamine-induced turning behaviour.

2.1.2. Experiment 2

Human embryonic VM tissue obtained at two dissection sessions was either prepared for immediate transplantation (FRESH; $n = 11$) or frozen 2–4 h after dissection (FROZEN; $n = 9$). Grafted animals were tested for amphetamine-induced motor asymmetry prior to and 15 weeks following transplantation. Quantitative and morphological analyses of the DA neuron-rich grafts were performed on sections immunostained with TH-antiserum for assessment of DA neurons and an antibody against human neurofilament to reveal general fiber outgrowth into the rat striatum.

2.1.3. Experiment 3

Human embryonic diencephalic tissue for Expt. 3 was dissected from same embryos as the VM tissue of Expt. 2. Animals were transplanted 3–5 h after dissection (DIENC; $n = 7$) and tested for functional graft effects 15 weeks later. Sections through the grafts were stained with Nissl stain for general graft morphology. Again TH- and HNF-immunocytochemistry were used to specifically evaluate neuron survival and fiber outgrowth from the grafts.

2.2. Animals and lesion surgery

Female Sprague–Dawley rats (B & K Universal AB, Sollentuna, Sweden; 200–220 g body weight) were subjected to unilateral 6-hydroxydopamine (6-OHDA, Sigma) lesions of the right ascending mesotelencephalic pathway as described previously [9].

2.3. Behavioural testing

Twelve–16 days following the 6-OHDA lesion, the amphetamine-induced (2.5 mg/kg i.p.) rotational behaviour was monitored in automated 'rotometer bowls' [47] over a period of 90 min. Rats with a mean rotational score of at least 6.5 net turns/min ipsilateral to the lesion side were selected for grafting, which is consistent with a >98% depletion of striatal DA and does not permit spontaneous recovery of the asymmetric behaviour [41,42]. Motor asymmetry tests were repeated at 15 weeks after transplantation surgery for Expts. 2 and 3 [33].

2.4. Donor tissue dissection and preparation

In order to establish the relative position of the mesencephalic and the diencephalic TH-immunoreactive cell groups, sagittal sections from one human embryo aged 6.5 weeks post-conception were processed for TH-immunocytochemistry. The whole embryo was immersion-fixed in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB, pH 7.4) overnight. It was then dehydrated in 20% sucrose/PB, sectioned sagittally at 40 μm thickness on a freezing microtome and stained according to the protocol described below (see section 2.8.).

2.4.1. Experiment 1

In Expt. 1, ventral mesencephalic tissue pieces were dissected out from 3 embryos aged 6, 6.5 and 7 weeks post-conception and divided along the midline into two equally sized pieces. All the tissue was initially stored for 24 h in hibernation medium at 4°C [26,39] before being divided into two equivalent batches. One batch of tissue was then grafted (HIB), whereas the other was frozen (HIB-F) for 3 days before thawing and transplantation (for details see section 2.5.).

The tissue batch allocated to the HIB group was grafted into nine animals according to a protocol modified from Björklund et al. [4]. Briefly, the donor tissue was transferred from the hibernation medium into a solution of 0.05% deoxyribonuclease (DNase; Sigma) in sterile 0.6% glucose/saline. Each half VM was further cut into 4 equally sized pieces using iridectomy scissors. After a 20 min incubation at 37°C in 0.1% trypsin (crude type II, Sigma), the tissue was rinsed 4–5 times with 20% fetal calf serum (FCS) in DNase/glucose/saline. A cell suspension rich in tissue aggregates was prepared in a final volume equivalent to 40 μl per complete VM, by gentle trituration using fire-polished Pasteur pipettes of decreasing bore diameters (1.0–0.3 mm).

2.4.2. Experiment 2

Human mesencephalic tissue was obtained at two dissection sessions from a total of 3 embryos (aged 6 and 7.5 at the first and 7 weeks post-conception at the second dissection session). Each VM piece was divided along the midline as in Expt. 1. One half of the tissue was immediately prepared into a cell suspension following the same protocol as in Expt. 1. The final volume was approximately 50 μl per complete VM. The remaining pieces of VM tissue were taken for cryopreservation according to the protocol described in a later section (see section 2.5.).

2.4.3. Experiment 3

The diencephalon was dissected from 2 embryos (6 and 7.5 weeks post-conception, the same embryos as used in Expt. 2). For the dissection, cuts were made caudally at the mes–diencephalic junction; rostrally just anterior to the pituitary; and then 1 mm lateral to the midline on each side, resulting in a tissue piece measuring approximately 2×2 mm, with a thickness of around 1 mm. Each piece was further subdivided into 4 pieces and immediately prepared into a cell suspension following the same protocol as in Expt. 1. The final volume of the cell suspension was equivalent to 20 μl per complete diencephalon.

2.5. Freezing procedure

The VM tissue allocated to the HIB-F group of Expt. 1 was transferred from hibernation medium after 24 h at 4°C into a sterile 1.8 ml Nalgene cryotube filled with 1 ml of 10% FCS in 0.05% DNase/glucose/saline. The VM tissue allocated to the FROZEN group of Expt. 2 was transferred into the same medium immediately after dissection.

At a temperature of 4°C the probes were preincubated in increasing concentrations of dimethyl-sulphoxide (DMSO, Sigma; 2, 4, 6, 8 and 10% v/v) for 10 min per concentration step. In a final concentration of 10% DMSO, the samples were frozen using an electronically controlled freezing system (Planer Biomed, Cryo 10 series; Planer Products LTD): tissue was cooled down from 4°C to −30°C at a rate of 1°C/min, followed by a cooling step of 2°C/min down to −60°C and, finally, immediate immersion into liquid nitrogen.

After the respective storage periods at −196°C (3 days for Expt. 1, 8 days for Expt. 2) the samples were thawed quickly (1–2 min) in a 37°C water bath. The tissue was transferred into a 14 ml reaction tube where stepwise 1:1-dilution of the DMSO-containing medium was performed by adding the appropriate volumes of 20% FCS/DNase/glucose/saline every 10 min. The tissue was finally rinsed 4–5 times in this solution.

Cell suspensions were prepared by mechanical dissociation without prior trypsin incubation and grafted as described below (see section 2.6.).

2.6. Transplantation surgery and immunosuppression

Under equithesin anesthesia (3 ml/kg body weight, i.p.), 2 μl of the cell suspension were injected at each of two striatal transplantation sites using a 10 μl Hamilton microsyringe fitted with a steel cannula (inner diameter = 0.25 mm). With an injection rate of 1 μl/min the deposits were placed at the following coordinates in relation to bregma A: +0.7 mm, L: 2.3 mm and 3.2 mm to the right, V: 4.5 mm ventral to the dura, with the tooth-bar (TB) set at zero.

A daily immunosuppressive treatment of 10 mg/kg i.p. of cyclosporin A (Sandimmune, Sandoz, 50 mg/ml diluted to 10 mg/ml in sterile saline) combined with a daily administration of tetracycline (Terramycin, Pfizer) through the drinking water (20–50 mg/kg) started on the day of transplantation.

2.7. Perfusion, fixation and sectioning

Under chloral hydrate anesthesia (350 mg/kg), the rats were perfused through the ascending aorta with 20–40 ml 0.1 M PB (room temperature), followed by 250–300 ml of ice-cold 4% PFA in PB. After 8 h post-fixation, the brains were dehydrated in 20% sucrose/PB and cut at 30 μm thickness in sagittal (Expt. 1) or coronal planes (Expt. 2) on a freezing microtome.

Sections which were stained using free floating staining methods (TH and HNF immunocytochemistry) were mounted on gelatin-coated slides after the staining procedure, left to dry overnight, dehydrated in ascending alcohol concentrations and cleared in xylene. Coverslips were mounted using DePeX (Serva, Germany).

2.8. Tyrosine-hydroxylase-immunocytochemistry and morphological assessment

Every third section was processed for free-floating TH-immunocytochemistry as described by Sauer et al. [40].

Tyrosine-hydroxylase positive graft cells were microscopically counted on every third section under bright field illumination and an approximation of total graft cell numbers was calculated according to the formula of Abercrombie [1]. Graft volumes were determined by making camera lucida drawings of the graft outlines, cutting them out, and weighing them to the nearest milligram before transforming paper weight to graft volume on the basis of section thickness and section frequency. For the assessment of cell sizes, parts of the grafts containing TH-positive neurons were selected randomly from 10 FRESH and 4 FROZEN rats in Expt. 2. Microscopic images of TH-positive neurons were digitized at 40× magnification using the Image 1.41 program with a frame grabber card (Macintosh). The long and short axis of 13–49 neurons per rat were measured and statistically analysed.

2.9. Human neurofilament – immunocytochemistry

Selected sections from rats containing either fresh mesencephalic or diencephalic grafts were stained with a mouse primary antiserum specifically recognizing the polypeptide 70 K constituent of human neurofilaments (1:200 dilution in 2% NHS/0.3% Triton X-100/PB, 48h at 4°C; Serotec, England) after a 60 min preincubation in 10% normal horse serum (NHS)/PB. A Vectastain ABC system was used to detect the primary antibody with 3,3'-DAB as chromogen [48]. The sections were qualitatively analysed for fiber outgrowth into the host striatum using brightfield and darkfield microscopy.

Fig. 1. Photomicrograph of a parasagittal section through the brain of a human embryo, processed for tyrosine hydroxylase-immunocytochemistry. At an post-conceptional age of 6.5 weeks, the cell groups of the developing substantia nigra/ventral tegmental area (SN/VTA) form a dense cluster in the ventral midbrain. The mesencephalic flexure (MF) is used as a landmark for dissection of this cell group. In the diencephalon (D), the developing hypothalamic TH-immunoreactive cell groups can be observed. Arrows indicate rostrally projecting TH-immunoreactive fibers forming the mesotelencephalic bundle. Bar = 400 μm.

2.10. Cresyl violet stain

Selected sections of diencephalic grafts were mounted on slides, left to dry, rinsed in destilled water and incubated in Cresyl violet for 25 s. Dehydration was performed as described for the free-floating staining methods.

3. Results

3.1. Tyrosine hydroxylase-immunoreactive cell groups in the human embryonic brain

In sagittal sections from the human embryo aged 6.5 weeks, a dense group of TH-immunoreactive neurons was found in the ventral mesencephalon (Fig. 1). These cells displayed an immature morphology, with small ovoid cell bodies and short, varicose neurites. Numerous TH-immunoreactive fibres were observed projecting rostrally from the ventral mesencephalon.

In the diencephalon, large numbers of scattered TH-immunoreactive cells were observed. Rostrally, weakly immunoreactive cells extended almost as far as the striatal anlage. More caudally (approximately halfway between the VM cell groups and the striatal anlage), a dorsal and a ventral aggregation of diencephalic TH-immunoreactive cells could be distinguished (Fig. 1). In general, cells in the diencephalon displayed a weaker TH-immunoreactivity, but somewhat larger cell bodies than cells in the developing mesencephalon.

3.2. Behavioural testing

3.2.1. Experiment 2

The rats were tested for amphetamine-induced motor asymmetry both prior to and 15 weeks after transplantation surgery. There was no significant difference in pre-transplantation motor asymmetry scores between the FRESH and the FROZEN groups (unpaired two-tailed Student's t-test, $P > 0.28$). Two-way analysis of variance (ANOVA) revealed a significant group × time interaction ($F_{1,18} = 8.87$, $P < 0.01$). Motor asymmetry scores following transplantation were significantly lower in the FRESH group when compared to FROZEN (unpaired two-tailed Student's t-test, $P <$ 0.01). Within the FRESH group 8 out of 11 rats displayed a more than 50% reduction in motor asymmetry and the mean rotation score post-transplantation was significantly lower than before grafting in this group (Fig. 2; paired two-tailed Student's t-test, $P <$ 0.01). Three of these animals performed more contralateral than ipsilateral body turns per minute, indicating complete reversal of motor asymmetry. In the FROZEN group, one out of 9 rats displayed more than a 50% reduction (96% less than pre-graft value) in motor asymmetry (Fig. 2). Spontaneous reductions in amphetamine-induced rotational asymmetry of this magnitude are generally not observed [41,42]. Upon subsequent morphological analysis (see below) this rat was found to contain the largest graft in the FROZEN group. However, as a whole, there was no statistical reduction in motor asymmetry in the FROZEN group compared to pre-transplantation (paired two-tailed Student's t-test, $P = 0.56$).

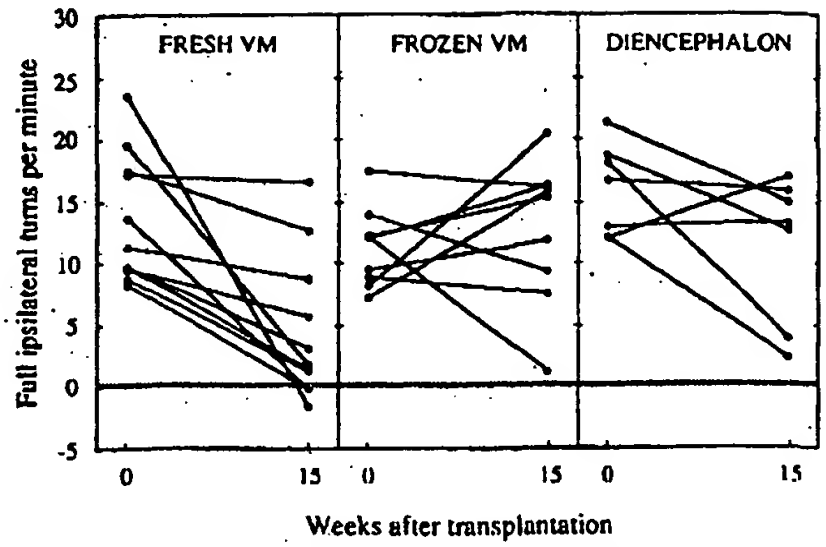


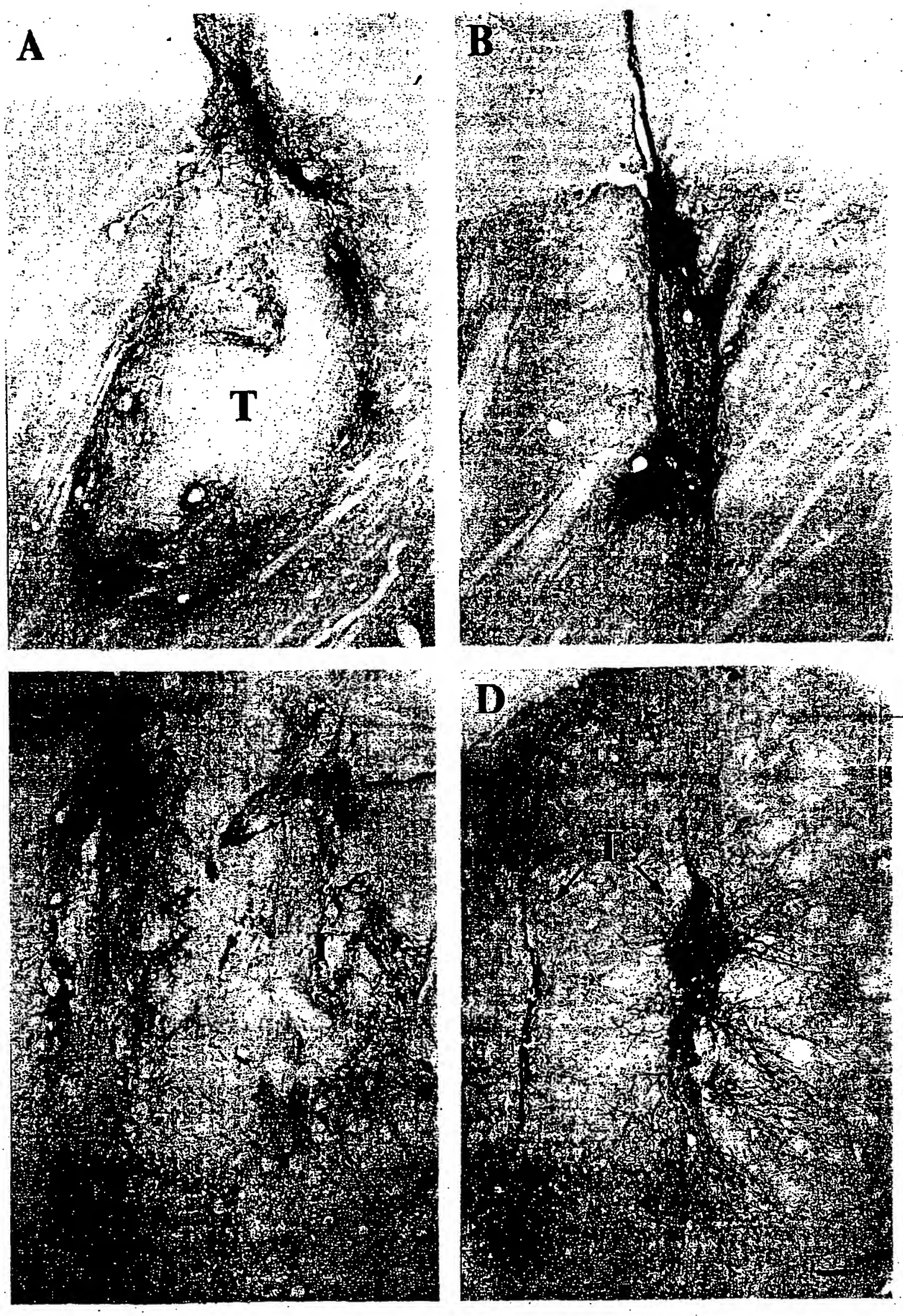
Fig. 2. The individual amphetamine-induced motor asymmetry scores are shown for the rats grafted with FRESH or FROZEN VM tissue of Expt. 2 and with DIENC tissue of Expt. 3. The rats were tested for rotational behaviour prior to and 15 weeks following transplantation. A significant reduction of net rotational scores was only observed in the FRESH VM group (paired two-tailed Student's t-test, $P < 0.01$).

3.2.2. Experiment 3

Among the rats grafted with diencephalic tissue, 2 out of 7 displayed substantial functional effects at 15 weeks post-grafting (Fig. 2); the individual motor asym-

→

Fig. 3. In the upper panels are displayed photomicrographs of sagittal sections through the striatum of two rats grafted with either HIB (A) or HIB-F (B) VM tissue of Expt. 1. TH-immunocytochemistry reveals HIB grafts rich in TH-positive cells which are mainly located close to the graft-host border with the center of the transplant being devoid of neurons. HIB-F grafts display dense TH-immunoreactive cell clusters. The average graft volumes of the HIB-F group were reduced to about 50% of HIB graft volumes. In the lower panels are displayed photomicrographs of coronal sections through the striatum of two rats transplanted with either FRESH (C) or FROZEN (D) VM tissue of Expt. 2. The mean number of TH-immunoreactive neurons in the FROZEN group was significantly reduced to 9% of the DA cell number in FRESH transplants. However, long TH-immunoreactive neurites were seen to extend into the host striatum in cases when the FROZEN VM grafts survived. The rat with the FRESH graft (C) displayed a 113% reduction in motor asymmetry score, and contained a total of 7568 DA neurons. The FROZEN graft (D) containing 1712 DA neurons gave rise to a 96% reduction of rotational score in the host rat. Bar = 100 μm.


A
T
B
D

metry scores were reduced by 82% and 87% in these two behaviourally compensated animals. In contrast to fresh mesencephalic tissue (see Expt. 2), no contralateral turns were performed by any rat. The two rats with reductions in turning were the only ones in the diencephalon group which contained grafts exhibiting markedly TH-positive neurons upon subsequent morphological assessment (see below). Statistically, amphetamine-induced turning behaviour was not significantly reduced in the diencephalon-grafted group (paired, two-tailed Student's t-test, $P = 0.11$).

3.3. Morphological analysis

3.3.1. Qualitative aspects

Upon inspection, the two intrastriatal graft tissue deposits were found to have merged in several specimens; thus, they were treated as one graft per animal for quantitative analyses of graft cell numbers and volumes.

Morphological analysis of the non-frozen VM groups (Expts. 1 and 2) revealed distinctly stained TH-positive neurons with their typical multipolar shape. Long axons in TH-positive fiber bundles extended into the host striatum. The cells were arranged either singly or in the form of small aggregates, which were aligned mainly at the graft-host interface leaving central parts of the grafts devoid of TH-staining (Fig. 3A,C). In contrast, cryopreserved grafts of both Expts. 1 and 2 displayed a higher density of TH-staining throughout. The TH-immunoreactive cells were often organized in dense clusters all over the graft so that a subdivision into central and peripheral parts was hardly possible. There was also marked TH-immunoreactive fiber outgrowth into the host striatum of a similar magnitude to that observed in the non-frozen transplants (Fig. 3B,D).

Moreover, FRESH mesencephalic grafts stained for HNF-immunocytochemistry revealed a dense fiber network within the grafts (Fig. 4D) and an extension of fibers into the host striatum.

3.3.2. Experiment 1 – quantification

Due to the high density of TH-immunoreactive neurons within the transplants, quantitative analysis of graft DA cell numbers could not be performed in this experiment. This high density of TH-staining within the whole graft tissue was probably related to the short survival time of the grafts, which is in agreement with previous observations [39]. Surviving grafts were found in all rats of the HIB and the HIB-F groups. In the HIB-F group there was a non-significant trend for a 50% reduction of the total graft volume (0.36 ± 0.9 mm^3, mean ± S.E.M.; $n = 5$) as compared to the HIB group (0.7 ± 0.19 mm^3, mean ± S.E.M.; $n = 8$) (Table 1).

3.3.3. Experiment 2 – quantification

Surviving transplants were found in 10 out of 11 rats of the FRESH group and in 6 out of 9 animals grafted with cryopreserved tissue. In the cases being devoid of transplants, the needle tract was filled with numerous yellow-brown cells that resembled macrophages. These animals were still included for the statistical assessment of graft cell numbers and volumes.

In the FRESH group, a mean number of 2827 ± 775 (mean ± S.E.M.; $n = 11$) TH-positive neurons per rat was yielded, whereas only 265 ± 186 ($n = 9$) DA neurons survived transplantation after the freeze-storage. Accordingly, the relative survival rate of FROZEN DA neurons was significantly decreased to 9% of FRESH controls (unpaired Student's t-test, $P < 0.01$). Measurement of graft volumes indicated a relative graft survival of 6% for the FROZEN (0.055 ± 0.03 mm^3, mean ± S.E.M.; $n = 9$) group when compared to the FRESH (0.947 ± 0.23 mm^3, mean ± S.E.M.; $n = 11$) group (Table 1). Even when eliminating the rats with non-surviving grafts from the analysis, the FROZEN group only contained a mean of 397 ± 269 ($n = 6$) cells compared to a mean of 3110 ± 798 ($n = 10$) for the FRESH group. The rat with the largest graft in the FROZEN group contained 1712 TH-positive neurons and was identical to the one rat that displayed a graft-induced reduction in rotational asymmetry (see above; Fig. 3D).

3.3.4. Experiment 3 – quantification

In Cresyl violet stained sections there were clearly distinguishable diencephalic grafts in the host striatum, both in behaviourally compensated and non-compensated animals (Fig. 4A). The grafts contained numerous large perikarya with a neuron-like morphology. Tyrosine hydroxylase-immunoreactivity was present in

→

Fig. 4. In A is displayed a photomicrograph of a DIENC tissue transplant in a rat that displayed a 85% reduction in motor asymmetry scores. Cresyl violet staining of the coronal section reveals several large grafted cells with a typical neuron-like morphology. Bar = 50 μm. Panel B depicts tyrosine hydroxylase-immunocytochemistry on a coronal section of a DIENC graft shows few distinctly stained DA neurons within large grafts. The morphology of the TH-positive cells is comparable to that of the TH-positive neurons found in mesencephalic grafts (see Fig. 3A), with the exception that the mesencephalic neurons tend to be more intensely TH-stained. The lower panels display coronal sections of both diencephalic (C) and FRESH mesencephalic (D) grafts which were immunostained with an antiserum against human neurofilament (HNF). HNF-positive fibers are seen to spread extensively within the transplants, whereas the fiber outgrowth into the host striatum is relatively sparse and reaches only a few hundred microns. Bar = 100 μm.

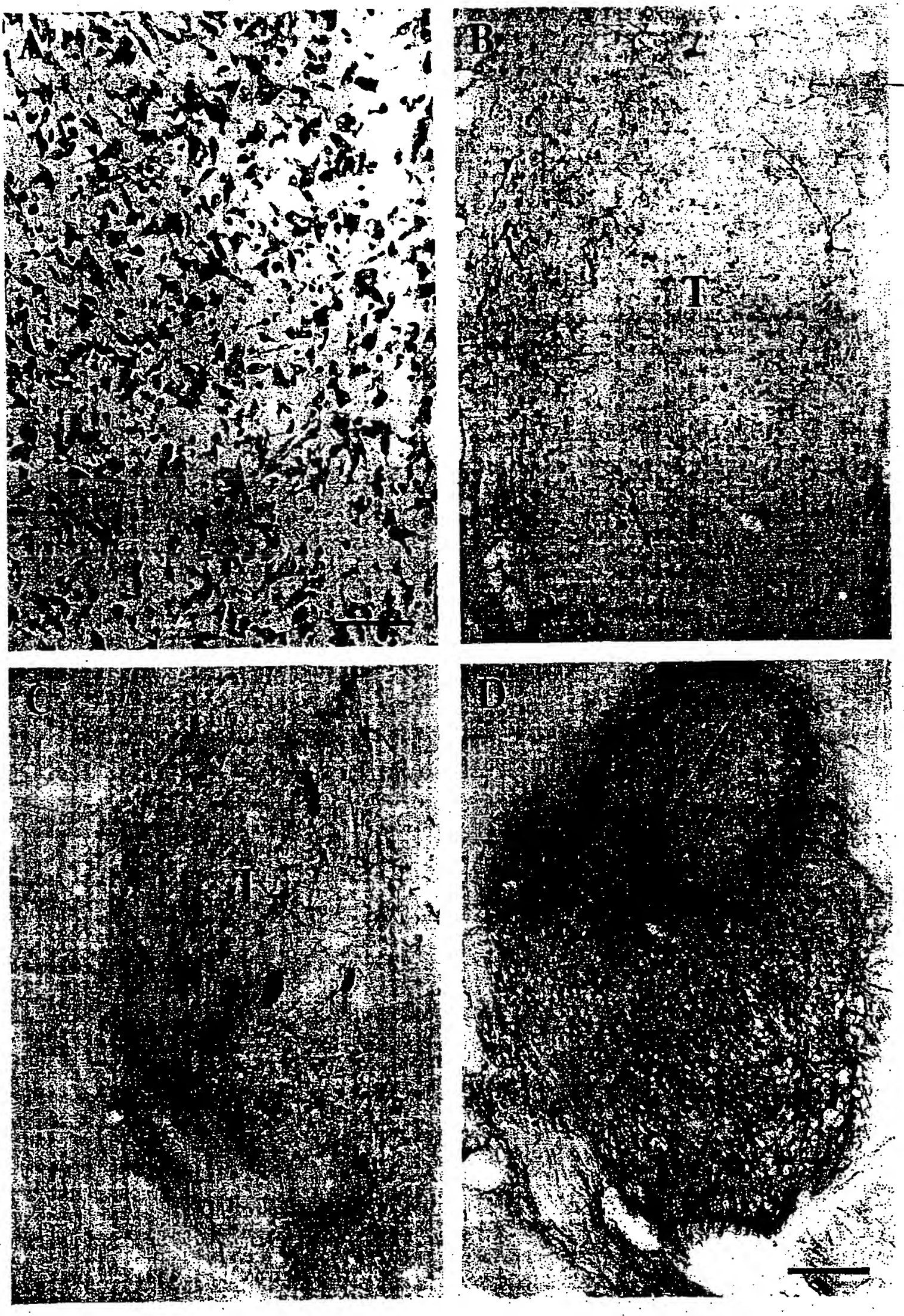

A
B
T
C
T
D

Table 1
Graft volumes (mm^3)

	NON-FROZEN	FROZEN
Expt. 1	0.71 (±0.19) ($n = 8$)	0.36 (±0.90) ($n = 5$)
Expt. 2	0.947 (±0.23) ($n = 11$)	0.055 (±0.03) * ($n = 9$)

Values are given as means ± S.E.M.
* denotes significant difference (unpaired Student's t-test, $P < 0.05$) between NON-FROZEN and FROZEN groups.

the transplants of the two rats which displayed functional compensation, whereas in the remaining 5 rats there was almost no TH-immunoreactivity in the grafts. The TH-positive neurons of diencephalic origin (Fig. 4B) were polydendritic in shape and morphologically comparable to mesencephalic TH-positive neurons (cf. Fig. 3C). However, since they displayed a weaker immunoreactivity than neurons dissected from the ventral mesencephalon, a quantification of cell numbers would have been very difficult and was, therefore, not performed. Human neurofilament-immunocytochemistry revealed extensive neurite growth within the graft and up to the graft–host border. However, fibers did not extend over long distances into the host striatum (Fig. 4C).

3.4. Cell size analysis

Assessment of the mean short and long axes of intrastriatally grafted human embryonic mesencephalic neurons revealed a significant difference (unpaired Student's t-test, $P < 0.001$) between the FRESH and the FROZEN groups (Table 2). The DA neurons in the FROZEN group were 27.8 ± 1.3 μm (mean ± S.E.M.) in the long and 10.3 ± 0.5 μm (mean ± S.E.M.) in the short axis, whereas the neurons found in the FRESH grafts exhibited a length of 35.6 ± 2.0 μm (mean ± S.E.M.) and a width of 14.4 ± 0.8 μm (mean ± S.E.M.).

Table 2
Dopamine neuron size (μm)

	NON-FROZEN ($n = 10$)	FROZEN ($n = 4$)
Long diameter	35.6 (±2.0)	27.8 (±1.3) *
Short diameter	14.4 (±0.8)	10.3 (±0.5) *

Values represent the means (±S.E.M.) of the average cell sizes for 4–10 rats per group.
* denotes statistical significant difference (unpaired Student's t-test, $P \leq 0.001$) between FRESH and FROZEN values.

4. Discussion

4.1. Cryopreservation

The cryopreservation procedure we used draws upon the experiences of others who have cryopreserved neuronal tissues in rat and non-human primate studies [11,12,16,24,25,40,45,46] or in human neuronal culture and transplantation experiments [34,35,38,43]. In the present study, the cooling rate was selected to be 1°C/min down to −30°C which is supposed to be the critical temperature range [10,13,23,24,43,44], and −2°C/min between −30°C and −60°C. Since the length of the storage interval following freezing does not seem to affect neuronal survival [2], the difference in the length of the cryopreservation intervals we chose is unlikely to have contributed to the differences in observed reductions in graft volumes of 49% and 94% between the cryopreserved groups in Expts. 1 and 2, respectively. Indeed, the survival rates observed following these relatively short storage intervals are probably representative also for storage periods of up to several months.

For practical reasons, we used a 24 h-hibernation interval prior to grafting or freezing in Expt. 1. As shown in our previous work, hibernation intervals of two days do not reduce DA cell yield after transplantation [39]. Interestingly, the DA cell yield after cryopreservation, relative to the respective control groups, in Expt. 1 was higher than in Expt. 2 which may suggest that a short hibernation interval prior to freezing may make the cells less susceptible to damage during the freezing procedure. It is conceivable, that the tissue habituates to a lowered metabolic state during the 24 h period in hibernation medium at 4°C. When thereafter the tissue is exposed to osmotic and ionic stress during freezing, this 'resting' state at 4°C could favour cell survival, since the transformation to a frozen state is less sudden. However, detailed biochemical and cryobiological investigations need to be performed to test this hypothesis.

4.2. Graft survival

Our published results obtained with rat mesencephalic tissue [40] show that cryopreservation can result in a decrease of cell numbers to about 1/3 of non-frozen control values, which we have confirmed also when applying slightly modified freezing protocols (Frodl, Sauer, Kupsch, unpublished observations). The results of the present study, in which cryopreservation caused a 49–94% drop in graft volume and 91% reduction in DA cell number, might suggest that human embryonic brain tissue *per se* is more sensitive to the freezing procedure. However, in vitro experiments of cryopreserved human embryonic cortical tissue have

revealed a survival rate around 60% suggesting that the type of neurons studied is critical [43]. Regarding human mesencephalic DA neurons, there are no previous studies comparing the survival of fresh versus cryopreserved grafts. A report showing a 90% viability, based on Trypan blue exclusion of cryopreserved human mesencephalic dissociated cells, is difficult to compare with the present experiment since it does not specifically consider the survival of the DA neuron population after a transplantation procedure [36]. The rat which contained the largest graft in the FROZEN group (1712 TH-positive neurons; Fig. 3D) also exhibited a 96% reduction in amphetamine-induced rotational asymmetry, strongly suggesting that the cryopreserved graft exerted a behavioural effect in this rat [5,6,37]. The five other rats in the FROZEN group which contained surviving grafts possessed only 9–362 TH-positive neurons and did not display any marked changes in rotational behaviour. This is consistent with previous studies which have shown that it is necessary to have a minimum threshold number of surviving DA neurons in the graft for functional effects to appear in the amphetamine-induced rotation test [9].

4.3. Cell size analysis

In contrast to our previous findings with embryonic rat DA neurons [40], there was a statistically significant reduction in the size of TH-positive neurons following cryopreservation of human DA neurons. Assessment of long and short cell diameters of frozen neurons revealed a decrease of 22% and 28%, respectively, relative to the non-frozen DA cells. Cell size has been suggested to correlate to the extent of fiber outgrowth from grafted neurons [14]. As axonal outgrowth probably reflects the functional capacity of the transplants, the present data suggest that not only do a reduced number of cryopreserved DA neurons survive grafting, but each surviving neuron may also exhibit a reduced capacity to reinnervate the striatum.

4.4. Survival rates of grafted human embryonic DA neurons

In Expt. 2, each rat received 4 μl of graft tissue suspension and each embryonic VM was dissociated to give a total volume of around 50 μl. Thus, each rat was grafted with about 8% of one human embryonic VM. Since the mean number of surviving DA neurons in each rat was around 3000, we can estimate that 35,000–40,000 DA neurons survive xenografting to the rat striatum from each human embryonic mesencephalon. This figure compares favorably with our previous estimate of about 20,000–25,000 surviving DA neurons per human embryo [9]. The difference in cell number may be related to the use of different morphological techniques to detect the DA neurons, i.e. fluorescence histochemistry in our previous work and TH-immuncytochemistry in the present study. Indeed, from our previous work with grafted rat DA neurons it seems that in our hands TH-immunohistochemistry is a more sensitive technique since it detects up to 2–3 times more DA neurons than catecholamine histofluorescence [8,39].

In our previous study, we estimated that the survival rate of human DA neurons was approximately 5% [9]. This calculation was based on the figure of 20,000–25,000 surviving DA neurons and an estimate of around 450,000 DA neurons present in each human mesencephalon [22]. The data from the present study could suggest that the survival rate is higher, however, a recent morphological study by Pakkenberg et al. [32] suggests that one human mesencephalon may contain far in excess of 1 million DA neurons, since they indicate that one single substantia nigra contains 550,000 DA cells. Thus, the survival rate of xenografted human embryonic DA neurons can be approximated to 3–9%, suggesting that there is great room for technical improvement in the graft preparation.

4.5. Human embryonic diencephalic dopamine neurons

Freeman and coworkers [20] have described the development of the mesencephalic DA neuron groups in human embryos and discussed their use as graft donor tissue. Other DA-rich cell groups than those in the mesencephalon have not yet been systematically examined as a potential source of graft tissue. Two studies that have used MPTP-induced parkinsonism in the monkey or mouse have so far investigated survival of DA-rich olfactory bulb tissue [11] and hypothalamic tissue [50], respectively. Both studies report on poor graft survival, showing low numbers of weakly stained TH-positive neurons within the transplants. The grafted hypothalamic DA neurons did not display any effects on behaviour as assessed by amphetamine- and apomorphine-induced turning in the mice with MPTP-induced lesions [50]. This is consistent with results from cell culture studies indicating that the number of TH-immunoreactive neurons in rat diencephalic cell preparations is roughly 25–50% of the number found in mesencephalic preparations [3,5].

Using Falck-Hillarp catecholamine histofluorescence, Olson et al. [31] have described large catecholamine containing cell groups in the human *fetal* diencephalon. These groups were believed to partly correspond to the DA cell groups A 11, 12 and 13 as described in the rat [21]. According to our observations in an immunohistochemically processed human *embryo*, there are already at 6.5 weeks post-conception, large numbers of TH-containing neurons in the human diencephalon (Fig. 1). When this region was xeno-

grafted, we observed large implants in two out of seven rats. Cresyl violet staining showed that all the grafts were rich in cells with a neuron-like morphology (Fig. 4A) and sections selected for HNF-immunohistochemistry also showed an abundance of human neurofilaments within the grafts, with a few fibers innervating the host striatum (Fig. 4C).

In two rats which had displayed a reduction in amphetamine-induced motor asymmetry, the grafts were large and contained several TH-immunopositive neurons, as opposed to very few or no clearly stained cells in the other five rats. The results show that human diencephalic TH-containing neurons can survive grafting to the rat striatum and also suggest that they can reinnervate the host in a functional fashion. It is noteworthy that although the two rats containing grafts with strongly TH-stained neurons exhibited 80–85% reductions in motor asymmetry, neither rat performed any rotations contralateral to the grafted striatum under the influence of amphetamine. In the group grafted with non-frozen tissue in Expt. 2, all the rats exhibited at least a few amphetamine-induced contralateral turns, even in cases when the total motor asymmetry score for the whole 90 min test period revealed an net ipsilateral bias. We have previouly suggested that the performance of even a few amphetamine-induced turns contralateral to the 6-OHDA denervated side is an early and sensitive indicator of mesencephalic graft function [6], but the current data suggest that this does not apply for diencephalic DA neurons.

5. Conclusions

We conclude that: (1) only around 37 500 DA neurons survive xenografting from one human embryonic mesencephalon a few hours after dissection. (2) Cryopreserved human embryonic mesencephalic tissue grafts can, when the surviving grafts are large enough, exert functional effects when implanted into the DA-depleted rat striatum. However, human tissue may be more susceptible to damage when frozen than embryonic rat nigral tissue, which is reflected by poorer graft survival and a significant reduction of cell size of cryopreserved human neurons. Thus, we do not consider our cryopreservation procedure suitable for clinical trials. (3) Furthermore, embryonic diencephalic tissue – although not as efficient as VM tissue – could present an interesting source of donor tissue which may increase the number of DA neurons obtainable from one human embryo. Diencephalic grafts can in some cases display functional effects and contain TH-immunopositive neurons which extend fibers into the host striatum. However, dissection parameters, the optimal donor age and a quantitative analysis on DA neuron survival rates when grafting diencephalic tissue need to be defined before it can be considered for use in the clinical setting.

Acknowledgements

This work was supported by a grant from the Swedish MRC (B9412X10818-01A), The Medical Faculty at the University of Lund and 'Neurologiskt Handikappades Riksförbund'. EF was supported by grants from Boehringer Ingelheim and Medafor and is grateful to Prof. Oertel for the continous support of her work. The study is part of EF's doctoral thesis at the Ludwig-Maximilians-University of Munich.

References

[1] Abercrombie, M., Estimation of nuclear population from microtome sections, *Anat. Rec.*, 94 (1946) 239–247.
[2] Ashwood-Smith, M.J., The low temperature preservation of fetal cells. In R.G. Edwards (Ed.), *Fetal Tissue Transplants in Medicine*, University Press, Cambridge, 1992, pp. 299–321.
[3] Beyer, C., Pilgrim, C. and Reisert, I., Dopamine content and metabolism in mesencephalic cell cultures: sex differences and effects of sex steroids, *J. Neurosci.*, 11/5 (1991) 1325–1333.
[4] Björklund, A., Schmidt, R.H. and Stenevi, U., Functional reinnervation of the neostriatum in the rat by use of intraparenchymal grafting of dissociated cell suspensions from the substantia nigra, *Cell Tissue Res.*, 212 (1980) 39–45.
[5] Brundin, P., Isacson, O. and Björklund, A., Monitoring of cell viability in suspension of embryonic CNS tissue and its use as a criterion for intracerebral graft survival, *Brain Res.*, 331 (1985) 251–259.
[6] Brundin, P., Nilsson, O.G., Strecker, R.E., Lindvall, O., Åstedt, B. and Björklund, A., Behavioural effects of human fetal dopamine neurons grafted in a rat model of Parkinson's disease, *Exp Brain Res.*, 65 (1986) 235–240.
[7] Brundin, P. and Björklund, A., Survival, growth and function of dopamine neurons grafted to the brain. In F.J. Seil, E. Herbet and B.M. Carlson (Eds.), *Progress in Brain Research, Vol. 71*, Elsevier, Amsterdam, 1987, pp. 293–208.
[8] Brundin, P., Barbin, G., Strecker, R.E., Isacson, O., Prochiantz, A. and Björklund, A., Survival and function of dissociated rat dopamine neurones grafted at different developmental stages or after being cultured in vitro, *Dev. Brain Res.*, 39 (1988) 233–243.
[9] Brundin, P., Strecker, R.E., Widner, H., Clarke, D.J., Nilsson, O.G., Åstedt, B., Lindvall, O. and Björklund, A., Human fetal dopamine neurons grafted in a rat model of Parkinson's disease: immunological aspects, spontaneous and drug-induced behaviour, and dopamine release, *Exp. Brain Res.*, 70 (1988) 192–208.
[10] Chanaud, C.M. and Das, G.D., Growth of neural transplants in rats: effects of initial volume, growth potential, and fresh vs. frozen tissues, *Neurosci. Lett.*, 80 (1987) 127–133.
[11] Collier, T.J., Redmond, D.E., Sladek, C.D., Gallagher, M.J., Roth, R.H. and Sladek, J.R., Intracerebral grafting and culture of cryopreserved primate dopamine neurons, *Brain Res.*, 436 (1987) 363–366.
[12] Collier, T.J., Sladek, C.D., Gallagher, M.J., Blanchard, B.C., Daley, B.F., Foster, P.N., Redmond, D.E., Roth, R.H. and Sladek, J.R., Cryopreservation of fetal rat and non-human primate mesencephalic neurons: viability in culture and neural

transplantation. In D.M. Gash and J.R. Sladek (Eds.), *Transplantation in the Mammalian CNS, Progress in Brain Research, Vol. 78*, Elsevier, Amsterdam, 1988, pp. 631–636.

[13] Das, G.D., Houlé, J.D., Brasko, J. and Das, K.G., Freezing of neural tissues and their transplantation in the brain of rats: technical details and histological observations, *J. Neurosci. Meth.*, 8 (1982) 1–15.

[14] Doucet, G., Brundin, P., Descarries, L. and Björklund, A., Effect of dopamine denervation on survival and outgrowth from intrastriatal fetal mesencephalic grafts, *Eur. J. Neurosci.*, 2 (1990) 279–290.

[15] Engele, J., Pilgrim, C., Kirsch, M. and Reisert, I., Different developmental potentials of diencephalic and mesencephalic dopaminergic neurons in vitro, *Brain Res.*, 483 (1989) 98–109.

[16] Farrant, J. and Ashwood-Smith, M.J., Practical aspects. In M.J. Ashwood-Smith and J. Farrant (Eds.), *Low Temperature Preservation in Medicine and Biology*, Pitman, Kent, 1980, pp. 285–310.

[17] Freed, C.R., Breeze, R.E., Rosenberg, N.L., Schneck, S.A., Wells, T.H., Barrett, J.N., Grafton, S.T., Huang, S.C., Eidelberg, D. and Rottenberg, D.A., Transplantation of human fetal dopamine cells for Parkinson's disease: results at 1 year, *Arch. Neurol.*, 47 (1990) 505–512.

[18] Freed, C.R., Breeze, R.E., Rosenberg, N.L., Schneck, S.A., Krick, E., Qi, J-X., Lone, T., Zhang, Y-B., Snyder, J.A., Wells, T.H., Ramig, L.O., Thompson, L., Mazziotta, J.C., Huang, S.C., Grafton, S.T., Brooks, D., Sawle, G., Schroter, G. and Ansari, A.A., Survival of implanted fetal dopamine cells and neurologic improvement 12 to 46 months after transplantation for Parkinson's disease, *New Engl. J. Med.*, 327 (1992) 1549–1555.

[19] Freeman, T.B. and Kordower, J.H., Human cadaver embryonic substantia nigra grafts: effects of ontogeny, pre-operative graft preparation and tissue storage. In O. Lindvall, A. Björklund and H. Widner (Eds.), *Intracerebral Transplantation in Movement disorders, Vol. 4*, Elsevier, Amsterdam, 1991, pp. 163–170.

[20] Freeman, T.B., Spence, M.S., Boss, B.D., Spector, D.H., Strecker, R.E., Olanow, C.W. and Kordower, J.H., Development of dopaminergic neurons in the human substantia nigra, *Exp. Neurol.*, 113 (1991) 344–353.

[21] Fuxe, K. and Hökfelt, T., Catecholamines in the hypothalamus and the pituitary gland. In W.F. Ganong and L. Martini (Eds.), *Frontiers in Neuroendocrinology*, Oxford Univ. Press, London, 1969, pp. 47–96.

[22] German, D.C., Schlusselberg, D.S. and Woodward, D.J., Three-dimensional computer reconstruction of midbrain dopaminergic neuronal populations from mouse to man, *J. Neural Transm.*, 57 (1983) 243–254.

[23] Houlé, J.D. and Das, G.D., Freezing of embryonic neural tissue and its transplantation in the rat brain, *Brain Res.*, 192 (1980) 570–574.

[24] Jensen, S., Sørensen, T., Møller, A.G. and Zimmer, J., Intraocular grafts of fresh and freeze-stored rat hippocampal tissue: a comparison of survivability and histological and connective organization, *J. Comp. Neurol.*, 227 (1984) 558–568.

[25] Jensen, S., Sørensen, T. and Zimmer, J., Cryopreservation of fetal rat brain tissue later used for intracerebral transplantation, *Cryobiology*, 24 (1987) 120–134.

[26] Kawamoto, J.C. and Barrett, J.N., Cryopreservation of primary neurons for tissue culture, *Brain Res.*, 384 (1986) 84–93.

[27] Lindvall, O., Rehncrona, S., Brundin, P., Gustavii, B., Åstedt, B., Widner, H., Lindholm, T., Björklund, A., Leenders, K.L., Rothwell, J.C., Frackowiak, R., Marsden, C.D., Johnels, B., Steg, G., Freedman, R., Hoffer, B.J., Seiger, Å., Bygdeman, M., Strömberg, I., and Olson, L., Human fetal dopamine neurons grafted into the striatum in two patients with severe Parkinson's disease. A detailed account of methodology and a 6-month follow-up, *Arch. Neurol.*, 46 (1989) 615–631.

[28] Lindvall, O., Brundin, P., Widner, H., Rehncrona, S., Gustavii, B., Frackowiak, R., Leenders, K.L., Sawle, G., Rothwell, J.C., Marsden, C.D. and Björklund, A., Grafts of fetal dopamine neurons survive and improve motor function in Parkinson's disease, *Science*, 242 (1990) 574–577.

[29] Lindvall, O., Widner, H., Rehncrona, S., Brundin, P., Odin, P., Gustavii, B., Frackowiak, R., Leenders, K.L., Sawle, G., Rothwell, J.C, Björklund, A. and Marsden, C.D., Transplantation of fetal dopamine neurons in Parkinson's disease: one-year clinical and neurophysiological observations in two patients with putaminal implants, *Ann. Neurol.*, 31 (1992) 155–165.

[30] Nobin, A. and Björklund, A., Histochemical demonstration of monoamine neuron systems in the human fetal brain. In *Frontiers in Catecholamine Research*, Pergamon, Great Britain, 1973, pp. 677–681.

[31] Olson, L., Boréus, L.O. and Seiger, Å., Histochemical demonstration and mapping of 5-hydroxytryptamine- and catecholamine-containing neuron systems in the human fetal brain, *Z. Anat. Entwickl.-Gesch.*, 139 (1973) 259–282.

[32] Pakkenberg, B., Møller, A., Gundersen, H.J.G., Mouritzen Dam, A. and Pakkenberg, H., The absolute number of nerve cells in the substantia nigra in normal subjects and in patients with Parkinson's disease estimated with an unbiased stereological method, *J. Neurol. Neurosurg. Psychiatry*, 54 (1991) 30–33.

[33] Perlow, M.J., Freed, W.J., Hoffer, B.J., Seiger, Å., Olson, L. and Wyatt, R.J., Brain grafts reduce motor abnormalities produced by destruction of nigrostriatal dopamine system, *Science*, 204 (1979) 643–647.

[34] Redmond, D.E., Naftolin, F., Collier, T.J., Leranth, C., Robbins, R.J., Sladek, C.D., Roth, R.H. and Sladek, J.R., Cryopreservation, culture and transplantation of human fetal mesencephalic tissue into monkeys, *Science*, 242 (1988) 768–771.

[35] Redmond, D.E., Spencer, D., Naftolin, F., Leranth, C., Robbins, R.J., Vollmer, T.L., Roth, R.H., Bunney, B.S. and Kim, J.H., Cryopreserved human fetal neural tissue remains viable 4 months after transplantation into human caudate nucleus, *Soc. Neurosci. Abstr.*, 15 (1989) 123.

[36] Redmond, D.E., Leranth, C., Spencer, D.D., Robbins, R., Vollmer, T., Kim, J.H., Roth, R.H., Dwork, A.J. and Naftolin, F., Fetal neural graft survival, *Lancet*, 336 (1990) 820–822.

[37] Rioux, L., Gaudin, D.P., Gagnon, C., Di Paolo, T. and Bédard, P.J., Decrease of behavioral and biochemical denervation supersensitivity of rat striatum by nigral transplants, *Neuroscience*, 44 (1991) 75–83.

[38] Robbins, R.J., Torres-Aleman, I., Leranth, C., Bradberry, C.W., Deutch, A.Y., Welsh, S., Roth, R.H., Spencer, D., Redmond, D.E. and Naftolin, F., Cryopreservation of human brain tissue, *Exp. Neurol.*, 107 (1990) 208–213.

[39] Sauer, H. and Brundin, P., Effects of cool storage on survival and function of intrastriatal ventral mesencephalic grafts, *Restor. Neurol. Neurosci.*, 2 (1991) 123–135.

[40] Sauer, H., Frodl, E.M., Kupsch, A., ten Bruggencate, G. and Oertel, W.H., Cryopreservation, survival and function of intrastriatal fetal mesencephalic grafts in a rat model of Parkinson's disease, *Exp. Brain Res.*, 90 (1992) 54–62.

[41] Schmidt, R.H., Ingvar, M., Lindvall, O., Stenevi, U. and Björklund, A., Functional activity of substantia nigra grafts reinnervating the striatum: neurotransmitter metabolism and [14C] 2-deoxy-glucose autoradiography, *J. Neurochem.*, 38 (1982) 737–748.

[42] Schmidt, R.H., Björklund, A., Stenevi, U., Dunnett, S.B. and Gage, F.H., Intracerebral grafting of neuronal cell suspensions. III. Activity of intrastriatal nigral suspension implants as assessed by measurements of dopamine synthesis and metabolism, *Acta Physiol. Scand. Suppl.*, 522 (1983) 19–28.

[43] Silani, V., Pizzuti, A., Strada, O., Falini, A., Buscaglia, M. and Scarlato, G., Human neuronal cell viability demonstrated in culture after cryopreservation, *Brain Res.*, 473 (1988) 169–174.

[44] Silani, V., Pizzuti, A., Strada, O., Falini, A., Pezzoli, G. and Scarlato, G., Cryopreservation of human fetal adrenal medullary cells, *Brain Res.*, 454 (1988) 383–386.
[45] Sladek, J.R., Collier, T.J., Haber, S.N., Roth R.H. and Redmond, D.E., Survival and growth of fetal catecholamine neurons grafted into the primate brain, *Brain Res. Bull.*, 1 (1986) 809–818.
[46] Sørensen, T., Jensen, S., Möller, A. and Zimmer, J., Intracephalic transplants of freeze-stored rat hippocampal tissue, *J. Comp. Neurol.*, 252 (1986) 468–482.
[47] Ungerstedt, U. and Arbuthnott, G.W., Quantitative recording of rotational behavior in rats after 6-hydroxydopamine lesions of the nigrostriatal dopamine system, *Brain Res.*, 24 (1970) 486–493.
[48] Wictorin, K., Brundin, P., Sauer, H., Lindvall, O. and Björklund, A., Long distance directed axonal growth from human dopaminergic mesencephalic neuroblasts implanted along the nigrostriatal pathway in 6-hydroxydopamine lesioned adult rats, *J. Comp. Neurol.*, 323 (1992) 475–494.
[49] Widner, H., Tetrud, J., Rehncrona, S., Snow, B., Brundin, P., Gustavii, B., Björklund, A., Lindvall, O. and Langston, J.W., Bilateral fetal mesencephalic grafting in two patients with Parkinsonism induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), *N. Engl. J. Med.*, 327 (1993) 1556–1563.
[50] Zuddas, A., Corsini, G.U., Barker, J.L., Kopin, I.J. and di-Porzio, U., Specific reinnervation of lesioned mouse striatum by grafted mesencephalic dopaminergic neurons, *Eur. J. Neurosci.*, 3 (1991) 72–85.